Effects of herbicides on both adaptive and acquired antibiotic resistance

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Abstract

Antibiotic resistance is an increasingly serious global health issue that will not be solved without serious and considered intervention. In order to effectively combat increasingly resistant bacteria, a better understanding of the factors influencing the development of antibiotic resistance is necessary. Previous work from this lab has shown that commercial herbicide formulations can induce adaptive antibiotic resistance in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Kurenbach *et al.*, 2015).

To investigate the breadth of this response, *Staphylococcus aureus* was exposed to the same set of commercial herbicide formulations and antibiotics and three additional antibiotics commonly used to treat *S. aureus* infections. The pattern of herbicide-induced changes in antibiotic tolerance was similar but not identical to those observed for *E. coli* and *S. enterica*. The magnitude of changes in minimum inhibitory concentration (MIC) was often smaller for antibiotics that were used in both sets of experiments, while the largest changes were observed for the new antibiotics. These effects were observed at herbicide concentrations below application rates and, in some cases, at concentrations within the maximum residue limits (MRLs) allowable in animal feed and human food as defined by the Codex Alimentarius Commission (Codex Alimentarius Commision, 2016).

Whether the adaptive responses to the herbicides can lead to shifts in the population frequency of acquired antibiotic resistance was also tested. Specific combinations of herbicide and antibiotic that caused either increases or decreases in antibiotic tolerance were investigated in more detail. In two combinations of herbicide and antibiotic, ciprofloxacin + Kamba and ciprofloxacin + Roundup, that caused adaptive resistance to the antibiotic an increased frequency of acquired resistance was observed in *S. enterica.* When

two strains of *E. coli* with differing antibiotic resistance were exposed to a combination of herbicide and antibiotic, tetracycline + Roundup or streptomycin + Kamba, that caused a decrease in antibiotic tolerance, increased selection in favour of the resistant bacteria was observed.

Abbreviations

2,4-d	2,4-dichlorophenoxyacetic acid
2,4-D	commercial formulation of 2,4-dichlorophenoxyacetic acid
ae	acid equivalent
Amp	ampicillin
AMPA	aminomethylphosphonic acid
ANOVA	Analysis of Variance
A site	aminoacyl site
C	Celsius
cfu	colony forming units
Cip	ciprofloxacin
Cm	chloramphenicol
DNA	deoxyribonucleic acid
EF-G	elongation factor G
EOP	efficiency of plating
EPSPS	5-enolpyruvylshikimate-3-phophate synthase
ESBL	extended-spectrum beta lactamase
FACS	fluorescence activated cell sorting
Fus	fusidic acid
g	grams
GDP	guanosine diphosphate
GTP	guanosine triphosphate
K	Kamba
Kan	kanamycin
L	litres
LB	Luria-Bertani broth
mg	milligrams
MIC	minimum inhibitory concentration
mL	millilitres
mM	millimoles per litre
MRL	Maximum Residue Limit

mRNA	messenger ribonucleic acid
MRSA	Methicillin Resistant *Staphylococcus aureus*
nm	nanometres
NZ	New Zealand
OD_{600}	optical density measured at a wavelength of 600 nm
Oxa	oxacillin
ppm	parts per million
RNA	ribonucleic acid
RND	resistance nodulation-cell division
rpm	revolutions per minute
SEM	Standard Error of the Mean
Str	streptomycin
Tet	tetracycline
tRNA	transfer ribonucleic acid
µg	micrograms
UK	United Kingdom
µl	microlitres
USA	United States of America
Vanc	vancomycin

Chapter One

Introduction

Previous work has shown that sub-lethal herbicide concentrations induce a multidrug resistance phenotype in Gram-negative potential human pathogens (Kurenbach *et al.*, 2015). In this thesis, I will challenge and extend hypotheses related to that foundational study. First, I have demonstrated that the effect of herbicides on resistance is also seen in at least one Gram-positive pathogen, *Staphylococcus aureus*. Second, I have tested how the response to a herbicide can lead to shifts in the population frequency of increased resistance to antibiotics.

Herbicides are a class of biocides, designed to kill plants. They have become increasingly common in modern society. They are used not only in the agricultural industry but also in urban environments such as households and parks. There are conflicting reports regarding the safety of herbicides with some reports showing effects on non-target organisms (Bukowska, 2006; González *et al.*, 2006; Williams *et al.*, 2000). Herbicide residues have been detected in many environments. From this it can be expected that a wide range of organisms are being exposed to these chemicals, often at sublethal concentrations (Battaglin *et al.*, 2014; Ensminger *et al.*, 2013). Sublethal herbicide concentrations have been shown to cause reversible changes in the antibiotic tolerance of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Kurenbach *et al.*, 2015). We hypothesised that this effect would occur in other species of bacteria.

Antibiotics are a class of biocide targeting bacteria. The widespread use of antibiotics has resulted in a strong selective pressure in favour of antibiotic resistant bacteria to the extent that many antibiotics are now becoming obsolete (Beović, 2006). This, coupled with the lack of investment in the development of new drugs, has lead to an increasingly serious global health issue (The Review on Antimicrobial Resistance, 2015).

Antibiotic resistance can be grouped into three categories: intrinsic, acquired and adaptive resistance (Fernández *et al.*, 2011). Intrinsic resistance is a trait that is ubiquitous in a species of bacteria and is independent of antibiotic selective pressure or horizontal gene transfer (Cox & Wright, 2013). An example of intrinsic resistance is the multi-drug resistant phenotype exhibited by many Gram-negative bacteria when exposed to various classes of clinically effective Gram-positive antibiotics (Cox & Wright, 2013). There is overlap between the categories of antibiotic resistance, for instance the multi-drug resistant phenotype above is caused by the Gram-negative outer membrane being impermeable to many molecules and the expression of efflux pumps that reduce intracellular drug concentrations (Cox & Wright, 2013; Nikaido, 1994). The increased expression of efflux pumps is also a form of adaptive resistance (Fernández *et al.*, 2011).

Acquired resistance occurs when formerly susceptible bacteria become resistant through a genetic change (Fernández *et al.*, 2011). This change could be the result of a mutation or horizontal gene transfer mechanisms such as conjugation, transformation or transduction (Van Hoek *et al.*, 2011). Horizontal gene transfer generally results in high-level antibiotic resistance, sometimes to more than one drug, being passed on (Levy & Marshall, 2004). Some mutations are capable of conferring high-level antibiotic resistance, however the majority of mutations result in low-level antibiotic resistance (Levy & Marshall, 2004).

Nevertheless, these low-level mutations can accumulate, ultimately resulting in high levels of antibiotic resistance that can be passed on to daughter cells (Fernández *et al.*, 2011; Levy & Marshall, 2004).

Adaptive resistance is characterised by the induction of resistance to one or more antibiotics in response to the presence of an environmental signal (Fernández *et al.*, 2011). This change in antibiotic resistance generally returns to the original level when the inducing signal is removed, however in some cases the original level of resistance cannot be restored (Fernández *et al.*, 2011). There are a variety of factors that can induce adaptive resistance including the presence of sub-lethal concentrations of antibiotics or other toxic chemicals, changes in oxygen levels, pH, or the presence of ions (Fernández *et al.*, 2011). One aim of this thesis is to investigate how adaptive resistance can lead to acquired resistance.

Antibiotic residues have been found in a number of terrestrial and aquatic environments, often at sublethal concentrations (Baquero *et al.*, 2008; Martínez, 2008). Sublethal antibiotic concentrations have been shown to cause selection in favour of resistant bacteria and increase mutation frequencies (Gullberg *et al.*, 2011). Adaptive resistance has also been linked to increased frequencies of acquired antibiotic resistance (Shen *et al.*, 2011). Given the previously discovered links between herbicides and adaptive resistance, it is important to see if these changes can lead to acquired resistance.

1.1 Herbicides

Three commercial herbicide formulations were used in this project: Roundup, Kamba and 2,4-D with the active ingredients glyphosate, dicamba and 2,4-d respectively. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-d), a synthetic auxin, was the first commercial herbicide

used in agriculture starting in 1946 (Green, 2014; Heap, 1997). In 1960 another synthetic auxin herbicide, 3,6-dichloro-2-methoxybenzoic acid (dicamba) was developed (Green, 2014). Both of these herbicides are now used worldwide to control many dicotyledonous weeds in cereal crops (Cao *et al.*, 2010; Grossmann, 2010). In 1974 N-(phosphonomethyl)glycine (glyphosate) was introduced (Powles, 2008). Glyphosate inhibits the 5-enolpyruvyl-shikimate-3-phostphate synthase (EPSPS) enzyme (Sammons & Gaines, 2014; Steinrücken & Amrhein, 1980). This non-selective, broad-spectrum, post-emergence herbicide has since grown in popularity and is used in the crop fields, national parks, and urban areas of many countries (Beckie, 2011; Nandula *et al.*, 2005; Powles, 2008). However, in 2016, the European Commission recommended a ban on glyphosate-based products containing the co-formulant POE-tallowamine and that use in public parks, playgrounds, and gardens be minimised (European Commission, 2016).

Glyphosate forms a complex with the EPSPS enzyme, the subsequent inhibition of EPSPS reduces feedback inhibition in the shikimate pathway (Duke & Powles, 2008). This causes shikimic acid to accumulate and eventually impacts the synthesis of three aromatic amino acids: phenylalanine, tyrosine, and tryptophan (Duke & Powles, 2008; Sammons & Gaines, 2014). The exact mechanism through which glyphosate kills the plant is unclear however there are two general theories (Duke & Powles, 2008; Gao *et al.*, 2014). One theory is that the shortage of amino acids prevents necessary protein synthesis, while the other suggests that the inhibition of negative feedback in the shikimate pathway results in the increased production of shikimate-3-phosphate which limits the carbon available for other essential metabolic pathways (Duke & Powles, 2008). The shikimate pathway is also found in bacteria and fungi (Busse *et al.*, 2001).

Dicamba and 2,4-D act in a different manner, imitating the natural plant auxin indole-3-acetic acid (IAA) (Gleason *et al.*, 2011; Mithila *et al.*, 2011). These synthetic auxins are more stable inside the plant than IAA, allowing them to accumulate and eventually disrupt growth and kill the plant (Grossmann, 2010). How auxinic herbicides kill plants is still unknown despite their use in agriculture for over 60 years (Mithila *et al.*, 2011). The effects of these herbicides on the plants can be divided in to three phases: stimulation, inhibition and tissue decay (Gleason *et al.*, 2011; Grossmann, 2010). In the first phase ethylene and abscisic acid concentrations increase as metabolic processes are activated, causing tissue swelling and abnormal growth (Grossmann, 2010). In the next phase the growth of shoots and roots are inhibited while the accumulated abscisic acid reduces carbon dioxide formation and increases hydrogen peroxide levels within the plant (Bukowska, 2006; Grossmann, 2010). In the final phase the accumulated hydrogen peroxide causes damage to plant tissue with the chloroplasts, membranes and the vascular system breaking down (Gleason *et al.*, 2011; Grossmann, 2010). The final result is wilting and the eventual death of the plant (Grossmann, 2010).

The introduction of herbicides is reported to have decreased pre-harvest losses while simultaneously reducing cost and energy inputs (Heap, 1997). Farmers were able to reduce their dependence on other more expensive and time-consuming weed management strategies such as tillage, burning, and cover crops (Heap, 1997). The amount of pesticides sprayed on farms in New Zealand quadrupled from 1960 to 1985 with total pesticide use estimated to have increased by an order of magnitude from 1960 to 2006 (MacLeod & Moller, 2006). A side effect of the intensified use of herbicides has been dramatic increases in the prevalence of herbicide resistant weeds. In 1968 the first well-documented case of a

herbicide resistant weed was observed in the USA (Heap, 2014; Ryan, 1970). Worldwide there are now 404 unique cases of herbicide resistance with approximately 11 new cases being reported each year (Heap, 2014).

Herbicide-resistant genetically modified crops were first introduced in 1996 and further changed the patterns of herbicide use (Benbrook, 2012). An EPSPS homologue from *Agrobacterium sp.* that functions at higher glyphosate concentrations was inserted into crops to make them glyphosate-tolerant (Dill *et al.*, 2008; Nandula *et al.*, 2005). The largest manufacturer of such crops is Monsanto, selling them under the trade name Roundup Ready® and the product line includes glyphosate-tolerant soybean, cotton and maize (first released in 1996, 1997 and 1998 respectively) (Castle *et al.*, 2006; Givens *et al.*, 2009; Young, 2006). These crops quickly grew in popularity as farmers could now use a single glyphosate-based herbicide throughout the growing season instead of other more labour intensive weed management methods (Dill *et al.*, 2008; Service, 2007). For instance, in Argentina 99% of the soybean crop is now glyphosate-tolerant (Powles, 2008).

Roundup Ready® crop systems have increased demand for glyphosate-based herbicides while the use of other herbicides has fallen (Powles, 2008; Service, 2007). As a result there has been a strong selection pressure in favour of glyphosate resistant weeds (Nandula *et al.*, 2005; Young, 2006). As these weeds continued to emerge, glyphosate application rates increased in an attempt to control the invasive species (Benbrook, 2012). Crops that are resistant to more than one herbicide are being developed by agrochemical companies and, in some cases, are already available for use (Mortensen *et al.*, 2012). These include crops that are resistant to combinations of glyphosate, glufosinate and either dicamba or 2,4-D (Green, 2014; Wright *et al.*, 2010). Companies developing these crops suggest that 2,4-D or

dicamba should be applied in addition to the currently used concentrations of glyphosate (Mortensen *et al.*, 2012). The amount of 2,4-D and dicamba used is therefore likely to increase as the crops are commercialised and adopted by farmers (Mortensen *et al.*, 2012). Benbrook (2012) estimates that 2,4-D use on corn crops in the USA would increase by 30-fold from 2010 to 2019.

In the USA it was estimated that herbicide use increased by 239 million kilograms between 1996 and 2011 (Benbrook, 2012). As herbicides continue to be used in large volumes it is important to understand how their residues may be spreading throughout the environment along with any effects they may have on organisms other than the weeds they are developed to kill. Herbicides have the potential to move away from the areas they were applied and accumulate elsewhere, causing pollution in neighbouring environments (Chang *et al.*, 2011). Glyphosate, dicamba and 2,4-D are all water-soluble and as such are able move easily from agricultural environments into water, soil and atmospheric ecosystems (Battaglin *et al.*, 2014; Hua *et al.*, 2006).

Glyphosate is readily soluble in water and has been found to leach through surface and subsurface runoff into aquatic environments (Borggaard & Gimsing, 2008; Lane *et al.*, 2012). Glyphosate and its main breakdown product, aminomethylphosphonic acid (AMPA), have been detected in agricultural soil, surface water, and groundwater from urban catchments (Kremer & Means, 2009; Van Stempvoort *et al.*, 2014). Battaglin *et al.* (2014) found glyphosate in 39.4% and AMPA in 55% of water and sediment samples from across the USA. Ditches, drains, and rainwater samples had a particularly high frequency of identification (Battaglin *et al.*, 2014). Glyphosate residues were identified in 60% to 100% of air and rainfall samples in Mississippi and Iowa collected weekly in 2007 and 2008, although

concentrations were low (< 0.1 ppm) (Chang *et al.*, 2011). Glyphosate residues have also been detected in food, they were detected in 5.6% of bread samples taken in the UK in 2010 (Benbrook, 2012). Additionally, glyphosate-tolerant soybeans were found to have relatively high concentrations of glyphosate (3.3 ppm) while soybeans that were organic or conventionally treated had no detectable glyphosate (Bøhn *et al.*, 2014).

Dicamba binds poorly to soil particles, increasing the likelihood of leaching occurring (Cojocaru *et al.*, 2013). In addition, a study on herbicides used in Brazil found dicamba was one of the least adsorbed herbicides (Oliveira *et al.*, 2007). 2,4-d is weakly adsorbed by soil particles, increasing the potential for surface and ground water contamination (Hermosin *et al.*, 2006). Both dicamba and 2,4-d have been detected in water from a variety of sources including aquatic environments near farmland (Kuo *et al.*, 2012), surface water (Ensminger *et al.*, 2013), drinking water reservoirs (Donald *et al.*, 2007) and rain (Filkowski *et al.*, 2003). Herbicide residues have even been detected in carpet dust within homes (Colt *et al.*, 2004)

The application rates of herbicides recommended by manufacturers are set to ensure effective control of a wide range of weeds and prevent the emergence of herbicide-tolerant weeds (Crespo *et al.*, 2013). However, as herbicides can readily leach into other environments and have potential toxic effects on humans and animals, the concentrations of herbicides allowable on human food and animal feed are regulated (Horváth *et al.*, 2014). International agencies and governments set Maximum Residue Limits (MRLs) for pesticides they determine are in need of regulation (Horváth *et al.*, 2014; Shin *et al.*, 2011). The Codex Alimentarius Commission sets MRLs for traded commodities (Codex Alimentarius Commision, 2016). These limits are set based on the results of standardised trials with the

aim to reduce the risks to human health and the environment (Horváth *et al.*, 2014; MacLachlan & Hamilton, 2010).

Although herbicides are designed to kill plants they have the potential to affect non-target organisms in a number of ways including decreased growth rate, reduced respiration efficiency, DNA damage, and death (Bukowska, 2006; González *et al.*, 2006; Malatesta *et al.*, 2008; Soloneski & Larramendy, 2011). There are conflicting opinions regarding the safety of 2,4-d, dicamba and glyphosate. Some studies have determined that herbicides have negligible public health impacts if used as directed by regulatory agencies (Dill, 2005; Munro *et al.*, 1992). However, a number of other studies have demonstrated that these herbicides have effects on off-target organisms. From an ecological perspective, even small shifts in the abundance of one species can result in severe consequences for a whole community (Relyea, 2005).

The main focus in investigating off-target effects of herbicides has been on humans, other mammals and vertebrates. Studies have linked 2,4-d, dicamba and glyphosate to increased rates of non-Hodgkin's lymphoma (Chang *et al.*, 2011; McDuffie *et al.*, 2001). Outside of humans a wide range of effects have been observed. For example, 2,4-D has been proven to be toxic to *Labeo rohita* fingerlings (Arivu *et al.*, 2015), dicamba reduced pollinator visits to alfalfa (Bohnenblust *et al.*, 2016), and glyphosate reduced earthworms in soils (Casabé *et al.*, 2007; Correia & Moreira, 2010). Much less is known about the effects of herbicides on microorganisms.

The microbial communities in the soil represent an environment where organisms may be repeatedly exposed to herbicides over time. Glyphosate in particular has the potential to cause off-target effects on bacteria and fungi, inhibiting aromatic amino acid biosynthesis

(Busse *et al.*, 2001). At the recommended application rate glyphosate altered the soil microbial community in fields planted with glyphosate tolerant soybeans and maize (Kremer & Means, 2009). There was a shift towards the fungal *Fusarium* species and a decrease in *Pseudomonas* species detected (Kremer & Means, 2009). Banks *et al.* (2014) observed small changes in the microbial community of soils treated with glyphosate at application rate and proposed that herbicide could have long-term effects on community structure. Similarly, 2,4-d application has been linked to an increase in the presence of bacteria able to degrade it, which were already more common in agricultural soils than forest soils (Zabaloy *et al.*, 2010). Glyphosate application has been linked to an increased severity of fungal diseases in glyphosate-tolerant crops, potentially due to a weakening of plant defences that fungal pathogens take advantage of (Johal & Huber, 2009).

The toxic effects of herbicides on bacteria have been studied in a range of organisms. Glyphosate has been shown to inhibit the growth of beneficial bacteria in the chicken gut microbiota (Shehata *et al.*, 2013) and microbes that are used in the food industry (Clair *et al.*, 2012). Slight impacts on the growth of *E. coli* were observed upon exposure to two commercial formulations of glyphosate at concentrations far below application rates (0.09 ppm) (Botelho *et al.*, 2012). The amount of herbicide required to kill bacteria varies greatly depending on the organism in question and whether the active ingredient alone is tested or if it is in a commercial formulation. For instance, 100 ppm of Roundup or glyphosate alone was sufficient to inhibit the growth of *Enterococcus faecalis* however it took 10,000 ppm before a reduction in the survival of *Clostridium botulinum* was observed (Krüger *et al.*, 2013). The minimum inhibitory concentration (MIC) of Roundup was also determined to be 7,400 ppm ae for *E. coli* and 6,190 ppm ae for *S. enterica* (Kurenbach *et al.*, 2015). However,

a much higher concentration, 84,550 ppm, of pure glyphosate was required to inhibit the growth of all soil bacteria on solid growth media (Busse *et al.*, 2001). A pure form of 2,4-d was toxic to *E. coli* at concentrations ranging from 400 to 900 ppm depending on the strain (Balagué *et al.*, 2001). However, Kurenbach *et al.* (2015) found that 4648 ppm ae of a commercial formulation of 2,4-d was required to kill a different *E. coli* strain while a slightly higher amount, 5780 ppm ae, was necessary to kill *S. enterica*. A formulation of dicamba was found to be the least toxic of the three herbicides tested with MICs of 13,883 ppm ae and 14,485 ae for *E. coli* and *S. enterica* respectively (Kurenbach *et al.*, 2015).

While herbicides are toxic to bacteria at sublethal concentrations they can also change the response of bacteria to other biocides such as antibiotics. Kurenbach *et al.* (2015) showed that sublethal concentrations of three commercial herbicide formulations induced changes in the antibiotic tolerance of *E. coli* and *S. enterica* serovar Typhimurium. The three herbicides used were 2,4-D amine 800 WSG, with the active ingredient 2,4-dichlorophenoxyacetic acid, Kamba^{500}, which contains dicamba as the active ingredient, and Roundup weed killer, with glyphosate as the active ingredient (Kurenbach *et al.*, 2015). Depending on the specific combination of herbicide, antibiotic and bacterium, both increases and decreases in antibiotic tolerance were observed when the herbicide and antibiotic were present compared to the antibiotic only (Kurenbach *et al.*, 2015). There were some combinations that did not result in any significant change in antibiotic tolerance (Kurenbach *et al.*, 2015). The bacteria did not require any pre-exposure to the herbicides for the effects to occur (Kurenbach *et al.*, 2015). The purified active ingredients were also tested to determine if they were responsible for the effects caused by the commercial formulations (Kurenbach *et al.*, manuscript in preparation). In general, the changes in

antibiotic tolerance were in the same direction for the active ingredient as the commercial herbicide formulations, where there was a difference it was that no effect was seen for the active ingredient where there was an effect for the formulation or vice versa (Kurenbach *et al.*, manuscript in preparation). This indicates that the active ingredient tends to dominate the effect seen however it was not possible to directly compare the magnitude of the changes due to different concentrations of active ingredient used between the experiments (Kurenbach *et al.*, manuscript in preparation). Two other potential herbicide ingredients, the surfactants Tween80 and carboxymethylcellulose, were also tested. Where the surfactants caused an effect on the antibiotic tolerance of *S. enterica* it was always an increase in tolerance (Kurenbach *et al.*, manuscript in preparation). This suggests that although the active ingredient may be responsible for the main effect observed by Kurenbach *et al.* (2015) other adjuvants within the formulations may increase or decrease the strength of the effect.

1.2 Antibiotics and Resistance

Antimicrobial agents are molecules that are able to kill or suppress the growth of microorganisms (Milić *et al.*, 2013). Technically, antibiotics are antimicrobial agents that are naturally produced by organisms as opposed to a synthetic molecule or physical agent such as ultraviolet light. However, this distinction is not relevant for this thesis and so the term antibiotic will be used to refer to antimicrobial agents, either natural or synthetic, that kills or suppresses the growth of bacteria.

Bacteria that are resistant to antibiotics pose an increasingly serious threat to human health and survival. For example, multi-drug resistant and extremely drug resistant tuberculosis is responsible for 200,000 deaths per year (The Review on Antimicrobial Resistance, 2016). In

the United States alone, antibiotic resistant infections cause illness in two million people each year, resulting in at least 23,000 deaths (Centers for Disease Control and Prevention, 2013). This does not include the additional 250,000 illnesses and 14,000 deaths from *Clostridium difficile* infections (Centers for Disease Control and Prevention, 2013). Without intervention, these figures will continue to rise and many now routine medical procedures such as joint replacements and organ transplants will have a much higher rate of failure due to infection (The Review on Antimicrobial Resistance, 2016).

Increasing levels of antibiotic resistance have also been observed in New Zealand (Williamson & Heffernan, 2014). In 2012 there were 4,000 cases of infections by bacteria producing an extended-spectrum beta lactamase (ESBL), which confers resistance to all cephalosporin antibiotics and almost all penicillins (Thomas *et al.*, 2014; Williamson & Heffernan, 2014). A recent survey of *Staphylococcus aureus* infections in New Zealand found that the majority (71.4%) were acquired outside of healthcare facilities and that overall 8.9% of isolates were resistant to methicillin (MRSA) (Heffernan *et al.*, 2015). Of the susceptible isolates, 21.8% were resistant to fusidic acid and 8.7% were resistant to mupirocin (Heffernan *et al.*, 2015).

Even with the growing body of knowledge regarding the negative impacts of antibiotic resistance, they are still widely misused. For instance, in the USA up to 50% of antibiotics prescribed to humans are either unnecessary or not the optimal drug (Centers for Disease Control and Prevention, 2013). In addition antibiotics can be purchased without a prescription in certain countries and online (Mainous *et al.*, 2009; Okeke *et al.*, 1999). Antibiotic use in New Zealand is on the rise, with annual per capita consumption increasing by 43% from 2005 to 2012 (Thomas *et al.*, 2014). In addition, there has been an innovation

gap in the antibiotics market with no new drug classes developed from 1962 to 2000 (Fischbach & Walsh, 2009).

Antibiotics are not only used to treat infections in humans but also in agriculture for the treatment of sick animals, disease prophylaxis, crop dusting, and to promote growth (Ghosh & LaPara, 2007; Kemper, 2008; McEwen & Fedorka-Cray, 2002). In the USA in 2013, 14.8 million kg of antibiotics were sold for use in food-producing animals (United States Food and Drug Administration, 2013). It is estimated that 80% of the antibiotics used in the USA are used in food animals, while global estimates suggest that the amount of antibiotics consumed by livestock is double that used by humans (Aarestrup, 2012; Van Boeckel *et al.*, 2015). Global consumption of antibiotics is predicted to increase as developing countries move towards larger-scale farms (Van Boeckel *et al.*, 2015).

These antibiotics are mainly used prophylactically or as growth enhancers (Diarra & Malouin, 2014; Kemper, 2008). In 2006, the use of antibiotics to enhance growth was banned from commercial agriculture in Europe (Kemper, 2008). However, this practice is still common in some countries including the USA and China (Milić *et al.*, 2013). In the USA at least 17 classes of antibiotics have been approved for use as growth promoters, with over 50% of the antibiotics used in animal feed identical or similar to the antibiotics used in human treatments (Key & McBride, 2014; Milić *et al.*, 2013). The pig farming industry in the USA in particular relies on the use of antibiotics as growth promoters (Key & McBride, 2014; Mackie *et al.*, 2006). These treatments are given repeatedly at sub-therapeutic doses creating an environment that promotes the emergence and spread of antibiotic resistant bacteria (You & Silbergeld, 2014).

Antibiotics are used in the agriculture industry for reasons other than to promote growth. They are used to control and prevent disease within a herd of animals, particularly when animals are kept in crowded conditions where infections can spread rapidly (Mackie *et al.*, 2006). For instance, virginiamycin, ceftiofur, and bacitracin are all approved for this use in Canadian poultry farming (Diarra & Malouin, 2014). Oxytetracycline has been used in the USA since the 1950s to prophylactically treat honeybee colonies, controlling *Melissococcus pluton* and *Paenibacillus larvae* infections (Tian *et al.*, 2012). Tylosin was also approved for this use in 2005 (Tian *et al.*, 2012). Antibiotics are also used in aquaculture, particularly salmon and trout farming, where fish are given antibiotics as part of their food (Cabello, 2006).

A large proportion of the antibiotics administered are not metabolised. It is estimated that as much as 70 to 90% of an antibiotic dose is excreted in animal urine or faeces (Dolliver & Gupta, 2008; Kwon, 2011; Mackie *et al.*, 2006). These antibiotic residues can then be transferred to other environments through a number of pathways. Pig effluent is commonly disposed of through land application in the USA and studies have found up to 46 mg/kg of tetracycline in pig manure (Hölzel *et al.*, 2010; Mackie *et al.*, 2006). Antibiotics can also be spread into waterways by the movement of water through contaminated soil (Kemper, 2008). In addition, unconsumed fish food and faeces that contain antibiotics can diffuse into the sediment and be washed further away by the current (Cabello, 2006). Urban areas are also a source of antibiotic contamination through effluent from hospitals and households and waste disposal from pharmaceutical companies (Manzetti & Ghisi, 2014). Antibiotics can also be released into waterways when wastewater treatments fail to efficiently remove them (Milić *et al.*, 2013). Studies conducted in Germany, Switzerland and

the USA found low concentrations of 30 different antibiotics in samples taken from surface, ground, and drinking waters (Kemper, 2008; Milić *et al.*, 2013).

There are increasing concerns regarding how the use of antibiotics in agriculture may select for resistant bacteria, which can be transferred to humans through the food chain or via direct contact (Heuer *et al.*, 2011; Kemper, 2008). The antibiotics used in this thesis are all clinically relevant in either human or veterinary medicine. Fluoroquinolones and aminoglycosides are some of the most commonly prescribed antibiotics in human medicine while β-lactams and tetracyclines are common in veterinary medicine (Milić *et al.*, 2013). Other antibiotics such as chloramphenicol, fusidic acid and vancomycin are also important in clinical practice (Falagas *et al.*, 2008; Kemper, 2008). The antibiotics used for this thesis have differing modes of action as described below.

Ampicillin and oxacillin are both members of the β-lactam family of antibiotics. These chemicals inhibit synthesis of the peptidoglycan layer of the cell wall and are bactericidal (Waxman & Strominger, 1983; Yao *et al.*, 2012). They bind to the active site of penicillin binding proteins (PBPs) and inhibit their activity (Yao *et al.*, 2012). PBPs are responsible for forming peptide crosslinks within the peptidoglycan layer that maintains turgor pressure and cell shape (Waxman & Strominger, 1983; Yao *et al.*, 2012). When the antibiotics inhibit these proteins, they are unable to form crosslinks eventually resulting in cell lysis (Yao *et al.*, 2012). Oxacillin is an antibiotic that specifically targets Gram-positive bacteria (Brown, 2001).

Chloramphenicol is a bacteriostatic antibiotic that reversibly inhibits bacterial protein synthesis (Schwarz *et al.*, 2004). It binds to the peptidyl transferase site of the 50S subunit of the bacterial ribosome (Schwarz *et al.*, 2004). This stops tRNA from reaching the correct

orientation, preventing the formation of peptide bonds between amino acids and ultimately inhibiting protein synthesis (Dunkle *et al.*, 2010).

Ciprofloxacin is a fluoroquinolone antibiotic that targets bacterial type II topoisomerases, DNA gyrase and topoisomerase IV (Aldred *et al.*, 2014). DNA gyrase introduces negative supercoils and releases torsional strain at replication forks (Aldred *et al.*, 2014; Hawkey, 2003). Topoisomerase IV is involved in untangling knots in the bacterial chromosome (Aldred *et al.*, 2014). The enzymes form a complex with DNA, making a staggered double-stranded break with covalent bonds to DNA on either side, before guiding another DNA duplex through and finally re-ligating the DNA (Aldred *et al.*, 2014; Hawkey, 2003). When fluoroquinolones bind to the enzyme-DNA complex there is a conformational change that prevents the re-ligation of the double-stranded DNA break (Cheng *et al.*, 2013; Hawkey, 2003). This stalls the replication complex and causes chromosome fragmentation (Cheng *et al.*, 2013). Fluoroquinolones are bacteriostatic at low concentrations and have a bactericidal effect at higher concentrations (Hawkey, 2003).

Fusidic acid is a narrow spectrum bacteriostatic antibiotic that is mainly active against staphylococci (Collignon & Turnidge, 1999). Fusidic acid inhibits bacterial protein synthesis by binding to the elongation factor G (EF-G) ribosome complex with either GDP or GTP (Collignon & Turnidge, 1999). It stabilises EF-G GDP on the ribosome and prevents further peptide elongation by inhibiting the GTPase function of EF-G (Collignon & Turnidge, 1999)

Kanamycin and streptomycin both belong to the aminoglycoside family of antibiotics (Kotra *et al.*, 2000). They bind to the aminoacyl site (A-site) of the 30S ribosomal subunit, which is responsible for the recognition of codons and anticodons (Kotra *et al.*, 2000). The antibiotics alter the conformation of the complex formed between the aminoacyl-tRNA and

the mRNA codon (Kohanski *et al.*, 2008), this interferes with tRNA recognition increasing the amount of mismatches that occur ultimately resulting in mistranslated proteins (Kotra *et al.*, 2000).

Tetracycline is a bacteriostatic antibiotic that inhibits protein synthesis in bacteria (Chopra & Roberts, 2001; Griffin *et al.*, 2011). In a similar manner to the aminoglycoside antibiotics, it binds reversibly to the 30S ribosomal subunit blocking the binding of aminoacyl-tRNA at the A site, preventing the elongation of peptides (Brodersen *et al.*, 2000; Chopra & Roberts, 2001).

Vancomycin is a glycopeptide antibiotic that inhibits cell wall synthesis in Gram-positive bacteria (Reynolds, 1989). Vancomycin interferes with the late stage of assembly of the peptidoglycan cell wall by preventing the transglycosylation reaction (Hiramatsu, 1998; Reynolds, 1989). This occurs when vancomycin interacts with the substrate of the transglycosylase shielding it from the active site of the enzyme (Hiramatsu, 1998).

1.2.1 Non-antibiotic chemical antimicrobial agents

Salicylate, the active component of aspirin, is normally used as a non-steroidal anti-inflammatory, however it has been shown to cause adaptive resistance in a range of circumstances (Price *et al.*, 2000). Rosner (1985) showed that exposure to sodium salicylate and acetyl salicylate caused an increase in the tolerance of *E. coli* to a range of antibiotics (chloramphenicol, tetracycline, ampicillin and nalidixic acid). A similar effect was shown with cephalosporin antibiotics (Foulds *et al.*, 1989). However, salicylate caused a decreased tolerance to aminoglycoside antibiotics (Aumercier *et al.*, 1990). The effect of salicylate on antibiotic tolerance is not restricted to *E. coli*. An increased tolerance to a range of

antibiotics has also been observed for *S. enterica* (Kurenbach *et al.*, 2015; Sulavik *et al.*, 1997), *S. aureus* (Gustafson et al., 1999), *Campylobacter jejuni* (Shen *et al.*, 2011), *Pseudomonas cepacia* (Burns & Clark, 1992), and *Serratia marcescens* (Berlanga & Vinas, 2000).

Salicylate has been shown to activate the *E. coli mar* operon leading to the expression of efflux pumps and changes in membrane permeability (Cohen *et al.*, 1993; Price *et al.*, 2000). The *mar* operon can confer increased tolerance to a wide range of antibiotics including: chloramphenicol, cephalosporins, penicillins, fluoroquinolones and nalidixic acid, rifampicin, and tetracycline but not aminoglycosides (Cohen *et al.*, 1988; Randall & Woodward, 2002). The *mar* operon has the ability to alter the expression of multiple genes within the bacterial chromosome; therefore changes in its regulation can cause resistance to a range of structurally dissimilar antibiotics, organic solvents and household disinfectants (Alekshun & Levy, 1999). This resistance arises in *E. coli* partly as a result of decreased influx, through down-regulation of the synthesis of OmpF, and increased efflux, through up-regulation of AcrAB-TolC synthesis, a multi-drug efflux pump (Alekshun & Levy, 1999; Cohen *et al.*, 1989; Cohen *et al.*, 1988).

Certain biocides have a similar effect on bacteria when sub-inhibitory concentrations are used. For instance, sub-lethal concentrations of three biocides common in the food industry (trisodium phosphate, sodium nitrite, and sodium hypochlorite) were shown to increase or decrease the tolerance of various *S. enterica* strains to a range of antibiotics with the largest effect seen for aminoglycoside and cephalosporin antibiotics (Molina-Gonzalez *et al.*, 2014). This effect was shown using a variety of multi-drug resistant clinical isolates of *S. enterica* and highlighted the issue of using biocides at sub-lethal concentrations throughout the food

production process, which could lead to successive selections for more resistant bacteria until a level of resistance is reached that poses a serious human health problem (Molina-Gonzalez *et al.*, 2014).

Cationic biocides are used in a number of areas to control the growth of microbes including: medical dressings, contact lens cleaning solutions, swimming pools, and many household cleaning products (Moore *et al.*, 2008). At sub-lethal concentrations, these too have been shown to increase the tolerance of bacteria to antibiotics and can cause a selective pressure that favours acquired resistance (Moore *et al.*, 2008; Webber *et al.*, 2015). This effect was shown using biocides such as quaternary ammonium compounds, halogenated tertiary amide compounds, and oxidative compounds which caused selection for multi-drug resistant *S. enterica,* often harbouring mutations in efflux pump repression genes (Webber *et al.*, 2015). These studies highlight the risk of sub-lethal concentrations of biocides, which can result in selection for bacteria that are resistant to antibiotics even though there may not be any change in resistance to the biocide (Webber *et al.*, 2015). Much of the current legislation surrounding these biocides is only concerned with the lethal effect that in-use concentrations have on target organisms (or the absence thereof). However, as these studies have shown, sub-lethal effects can have real consequences in the selection for antibiotic resistant bacteria that need to be considered. This is especially relevant given the increasing levels of biocides and antibiotics that are being found in both water and terrestrial environments, which are becoming reservoirs of antibiotic resistance genes (Baquero *et al.*, 2008; Martínez, 2008).

The effect of salicylate and related chemicals on antibiotic tolerance is often physiologically reversible, meaning that when the chemical is removed, tolerance levels return to pre-

exposed levels (Rosner, 1985). However, there has been some work carried out regarding the influence of salicylate on the frequency of acquired resistance through mutation. These mutants no longer require the presence of salicylate to survive antibiotic exposure. The frequency of ciprofloxacin resistant *C. jejuni* mutants was 70-fold higher when salicylate was present in plates containing 4 µg/mL ciprofloxacin than when it was not (Shen *et al.*, 2011). However, there was no difference observed at the lower concentrations tested, 0.625 µg/mL and 1.25 µg/mL (Shen *et al.*, 2011). A similar effect was shown using *S. aureus* where the frequency of ciprofloxacin resistant mutants increased by 100-fold when bacteria were exposed to salicylate with both 0.4 µg/mL and 0.6 µg/mL ciprofloxacin (Gustafson *et al.*, 1999). The concentrations of ciprofloxacin required to induce this response were much closer to the minimum inhibitory concentration (MIC) of *S. aureus* (two and three times MIC) than *C. jejuni* (32 times MIC) (Gustafson *et al.*, 1999; Shen *et al.*, 2011).

Another way in which antibiotic resistance evolves is through selection at low concentrations of the antibiotic. Zhao and Drlica (2001) proposed the idea of a "mutant selective window". This window is the area between the concentration of the antibiotic that killed susceptible bacteria (MIC^{susc}) and the concentration of the antibiotic that killed resistant bacteria (MIC^{res}) (Drlica, 2003). It was hypothesised that selection for resistant bacteria only occurred within this window and not at concentrations of the antibiotic below the MIC^{susc} (Drlica, 2003; Drlica & Zhao, 2007). However, recent studies suggest that these concentration ranges are far wider than previously believed (Gullberg *et al.*, 2011).

There is now evidence to suggest that selection in favour of more resistant organisms can occur far below the level at which antibiotics observably begin to inhibit the growth of susceptible bacteria (Andersson & Hughes, 2012; Gullberg *et al.*, 2011). These studies have

shown that sub-lethal concentrations of antibiotics, such as those that might be found in soils and other environments, can be enough to select for antibiotic resistance (Andersson & Hughes, 2012; Gullberg *et al.*, 2011). For example, a concentration of tetracycline that was one-hundredth of the MIC of the susceptible strain (15 ng/mL) was sufficient to cause selection in favour of a more resistant strain in a competition environment (Gullberg *et al.*, 2011). For ciprofloxacin, even lower concentrations, 100 pg/mL, caused selection in favour of ciprofloxacin resistant bacteria (Gullberg *et al.*, 2011). These concentrations are in the same range those detected in aquatic and soil environments (Kemper, 2008; Kümmerer, 2009). Further work was also carried out to determine how multiple inhibitory substances (antibiotics and heavy metals) may create a combination effect and widen these spaces of selection (Gullberg *et al.*, 2014). While this principle has been shown with a number of substances, there has yet to be any investigation into how herbicides that decrease antibiotic tolerance may cause selection in favour of resistant bacteria at antibiotic concentrations that without the herbicides do not have any selective effect.

1.3 Objectives of this study

There are two core aims of this study. Firstly, to determine if the adaptive changes in the antibiotic tolerance of *E. coli* and *S. enterica* that was observed upon exposure to herbicides would also extend to the more distantly related, Gram-positive, *S. aureus*. Secondly, to determine if the previously observed adaptive responses could result in selection in favour of acquired resistance.

S. aureus was chosen because it is an important human and animal pathogen. The species has been accumulating increasing levels of antibiotic resistance, making some strains difficult to treat (Fitzgerald, 2012; World Health Organization, 2014). *S. aureus* also has the

ability to move between livestock and humans, certain livestock-associated methicillin resistant *S. aureus* (MRSA) strains have already been shown to colonise humans (Fitzgerald, 2012). Finally, *S. aureus* represents a different potential exposure pathway to the herbicides. *S. aureus* could be exposed to herbicides when humans and animals, colonised in the nares and skin (Kluytmans *et al.*, 1997), touch herbicide covered surfaces or inhale herbicides whilst they are being sprayed.

S. enterica and *E. coli* were used to investigate the effects of physiologically reversible increases and decreases in antibiotic tolerance on the development of acquired resistance. These bacteria were used in order to maintain consistency with the original study that observed the adaptive effect. In addition both species can cause disease in humans and are becoming increasingly resistant to antibiotics (World Health Organization, 2014).

I had three primary research questions:

1. Does the Gram-positive bacterium, *Staphylococcus aureus*, respond to herbicide-induction of antibiotic tolerance?

2. Can the herbicide-induced adaptive resistance lead to an increased frequency of acquired resistance within *S. enterica* populations?

3. Can the herbicide-induced physiologically reversible decreases in antibiotic tolerance result in selection pressure in favour of more resistant *E. coli* populations?

Chapter Two

Investigating the antibiotic tolerance of *Staphylococcus aureus* following exposure to commercial herbicide formulations

2.1 Introduction

S. aureus is commonly found on the skin and nares of humans and animals (Fitzgerald, 2012; Wertheim *et al.*, 2005). Approximately 30% of people are carriers of *S. aureus* (Wertheim et al., 2005), which can cause many illnesses in humans ranging from minor skin infections to life threatening endocarditis, necrotising pneumonia and toxic shock syndrome (Fitzgerald, 2012; Lowy, 1998). Similarly in animals *S. aureus* infections can cause skin abscesses, mastitis and septicaemia (Fitzgerald, 2012). *S. aureus* is also becoming increasingly resistant to antibiotics MRSA considered a very serious public health threat (The Review on Antimicrobial Resistance, 2016; World Health Organization, 2014).

The change in antibiotic tolerance of *S. aureus* following exposure to 24 antibiotic and herbicide combinations was measured. Three herbicides and eight antibiotics were used. The three herbicides and five of the antibiotics (ampicillin, chloramphenicol, ciprofloxacin, kanamycin, and tetracycline) were used to maintain consistency with the previous study (Kurenbach *et al.*, 2015). The other three antibiotics (oxacillin, fusidic acid, and vancomycin) are commonly used in clinical practice to treat *S. aureus* infections. Oxacillin is a penicillinase-stable β-lactam antibiotic; resistance to this antibiotic is how MRSA strains are classified (Brown, 2001; Clinical and Laboratory Standards Institute, 2012). Vancomycin is a

glycopeptide antibiotic that is a mainstay of treatment for MRSA (Chen *et al.*, 2015). Increased vancomycin tolerance is significantly associated with failure to treat *S. aureus* bacteraemia (Britt *et al.*, 2017). Fusidic acid is a first line recommended agent for the treatment of topical impetigo in New Zealand (Williamson *et al.*, 2014). New Zealand has high rates of fusidic acid usage and resistance compared to other countries (Heffernan *et al.*, 2015; Williamson *et al.*, 2014). There has been an increase in fusidic acid resistance among MRSA in NZ over the past decade (Williamson *et al.*, 2014).

Previous work has shown that sub-lethal concentrations of commercial herbicide formulations can induce changes to the antibiotic tolerance of *Salmonella enterica* and *Escherichia coli* (Kurenbach *et al.*, 2015). Further work has since been performed to determine if the herbicide active ingredients or other adjuvants in isolation can also lead to changes in antibiotic tolerance (Kurenbach *et al.*, manuscript in preparation).

The aim of this work was to determine whether the Gram-positive bacterium, *Staphylococcus aureus,* would also exhibit changes in antibiotic tolerance following herbicide exposure. I will also report the minimum herbicide concentrations required to induce any of the observed changes. In the initial work by Kurenbach *et al.* (2015), some patterns were observed where both species of bacteria exhibited similar responses to the herbicide and antibiotic. I investigated if these patterns extended to a more distantly related species.

2.2 Methods

2.2.1 Bacterial strains, culture conditions and chemicals

Staphylococcus aureus strain ATCC25923 (Taylor *et al.*, 1983) was used in this study. The strain was stored long-term at -80°C in LB medium supplemented with 15% glycerol. Bacteria were maintained for up to one week on Luria-Bertani broth (LB) Agar, grown at 37°C overnight and subsequently stored at 4°C. Luria-Bertani base (Lennox-L-Broth Base, Invitrogen (USA)) and agar (Bacteriological Agar No.1, Oxoid (UK)) were used. Bacteria were cultured at 37°C with rotation at 215 rpm providing aeration before use in experiments.

The antibiotics used in these experiments were: ampicillin (amp, stock concentration – 100mg/mL), chloramphenicol (cm, 20 mg/mL), ciprofloxacin (cip, 10 mg/mL), fusidic acid (fus, 10 mg/mL), kanamycin (kan, 10 mg/mL), oxacillin (oxa, 10 mg/mL), tetracycline (tet, 5 mg/mL) and vancomycin (vanc, 10 mg/mL). Stock solutions were stored at -20°C and diluted appropriately for experiments. Ampicillin sodium salt was purchased from Applichem (Germany), kanamycin sulfate from Life Technologies (USA), ciprofloxacin hydrochloride from Pentex (USA), chloramphenicol, fusidic acid sodium salt, oxacillin sodium salt, tetracycline hydrochloride and vancomycin hydrochloride from Sigma-Aldrich (USA).

Three commercial herbicide formulations were used in this study: Kamba, Roundup, and 2,4-D. Kamba^{500} (Nufarm, New Zealand) contains the active ingredient dicamba as a dimethylamine salt (500 g/L). Roundup Weed Killer concentrate (Monsanto, Australia) has the active ingredient glyphosate, present as an isopropylamine salt (360 g/L). 2,4-D Amine 800 WSG (Agpro, NZ) contains the active ingredient 2,4-dichlorophenoxyacetic acid (2,4-d) as a dimethylamine salt (800 g/kg). Initially, concentrations were calculated in millimoles

per litre (mM) before conversion to parts per million acid equivalent (ppm ae), for comparability to other formulations. The 2,4-D pellets were dissolved in sterile water to give a solution with a final active ingredient concentration of 66.24 g/L (300 mM). Herbicides were stored at room temperature and added to the growth medium at the appropriate concentration when required.

2.2.2 Determining the effect of commercial herbicide formulations on antibiotic tolerance

A killing curve assay was performed to determine the effect of each herbicide formulation on the antibiotic tolerance of *S. aureus.* LB agar plates containing a range of antibiotic concentrations both in the presence and absence of a constant herbicide concentration were inoculated with bacteria. Prior to these experiments, a preliminary test was performed to determine the minimum inhibitory concentration (MIC) of each herbicide. LB agar plates containing increasing concentrations of the herbicide were inoculated with *S. aureus* and survival measured. The killing curve assay uses a sub-lethal herbicide concentration, approximately half of the concentration at which bacteria first began to exhibit signs of decreased growth. A control with LB agar + herbicide was included in each killing curve assay to ensure the herbicide alone had no effect on survival.

Liquid LB medium was inoculated with a colony of *S. aureus* then incubated with aeration at 37°C until the culture reached an optical density (OD_{600}) of approximately 1. The OD_{600} was determined using a spectrophotometer that measures the absorbance of the culture at a wavelength of 600 nm. All LB agar plates were freshly poured and dried in a laminar flow cabinet for at least an hour before inoculation.

Plates were inoculated using a 'spot plating' technique. The culture of *S. aureus* was serially diluted 10-fold to a final dilution of 10^{-6}. Each dilution was then used to inoculate the agar plates with 10µl drops to give final dilutions of 10^{-2} to 10^{-8} compared to the original culture. After the droplets had dried, plates were incubated at 37°C and examined daily for up to four days. Individual colonies were counted and colony-forming units per mL (cfu/mL) calculated based on the dilution where growth was observed. The cfu/mL count for each treatment plate was compared to the LB only control to calculate the efficiency of plating (EOP). The EOP is a measure of survival used normalise for variations in culture density between replicates (Figure 2.1). Each killing curve assay was performed three times using independent cultures of bacteria. The minimum inhibitory concentration (MIC) of an antibiotic or herbicide was defined as the concentration that caused a 1000-fold reduction in EOP compared to the LB control.

Figure 2.1 – Formula used to calculate Efficiency of Plating (EOP).

$$EOP = \frac{Cfu/mL\ on\ Treatment\ Plate}{Cfu/mL\ on\ LB\ Control\ Plate}$$

2.2.3 Determining the minimum herbicide concentration that induces an antibiotic response

The minimum herbicide concentration that induced a change in antibiotic tolerance was determined by a dose response assay. Only combinations of herbicide and antibiotic where a statistically significant difference in survival occurred with the herbicide present were used in this assay. An antibiotic concentration that caused a large difference in EOP was used for the dose response assay. This antibiotic concentration was kept constant and the herbicide concentration reduced until differences in EOP between the antibiotic only

treatment and the antibiotic and herbicide treatment disappeared. The maximum herbicide concentration was the concentration used in the killing curve assay.

Liquid LB medium was inoculated with a colony of *S. aureus* then incubated at 37°C with rotation providing aeration until the OD_{600} reached approximately 1. LB agar plates were freshly poured and dried for at least an hour before being inoculated with 10µl spots of *S. aureus* from a 10-fold dilution series. After inoculation, plates were incubated at 37°C for up to four days, with colonies counted each day until no new growth occurred. EOP was calculated as described in Figure 2.1. The minimum herbicide concentration that induced a 100-fold change in EOP when compared to the antibiotic only control was then tabulated.

2.2.4 Statistical Analysis

R was used for all statistical analysis (R Core Team, 2015). For the killing curve assay, a multifactor analysis of variance (ANOVA) was performed on the log-transformed EOP scores to test for synergistic effects of herbicides and antibiotics. The significance of the herbicide by antibiotic interaction term was evaluated in order to determine whether a dose response curve should be performed for any given herbicide and antibiotic combination. For this analysis, plots of residuals were utilised to test for any violations of the assumptions of normality and equality of variance. Contrasts across herbicide concentrations when antibiotic levels were fixed were used to determine the antibiotic concentrations with significant differences among herbicide treatments. The testInteractions function in the phia package in R was used to evaluate the contrasts (De Rosario-Martinez, 2015). For this procedure, a Bonferroni correction (Crawley, 2007) was performed within each experiment.

For the dose response assay, residuals from the standard ANOVA were not normally distributed. Therefore, the equivalent non-parametric test, a Kruskal-Wallis one-way ANOVA was performed to test for differences in log-transformed EOP scores among herbicide concentrations. A comparison between a null model where EOP is the same across all herbicide concentrations and an alternative model where EOP differs among some herbicide concentrations was performed to give a p-value, which is reported. All graphs were made using the lattice package in R (Sarkar, 2008).

2.3 Results

2.3.1 Some commercial herbicide formulations change the antibiotic tolerance of Staphylococcus aureus

S. aureus was exposed to 24 unique herbicide and antibiotic combinations. Antibiotic concentrations ranged from those that caused no observable effect on growth to those above minimum inhibitory concentrations (MIC). The MIC of each commercial herbicide formulation was also calculated (Table 2.1). Roundup had the greatest impact on survival of the three herbicides. It had an MIC of 170 ppm ae. The concentration of each herbicide used in the killing curve assay was below MIC, at a concentration where there was no effect on EOP (Table 2.1). Log-transformed EOP scores were used for the statistical analysis, calculation of standard error of the mean (SEM) and creation of graphs. The detection limit for this method was an EOP $\approx 1 \times 10^{-7}$, representing a detection range of approximately seven orders of magnitude. When antibiotic concentrations were low, EOP was close to 1, as the concentration approached and exceeded the MIC, the EOP eventually dropped to below the detection limit.

Table 2.1 – Herbicide concentrations used for killing curve experiments and minimum inhibitory concentrations.

Herbicide	MIC (ppm ae)	Concentration used (ppm ae)
2,4-D	2130 ± 120	914
Kamba	11580 ± 300	1371
Roundup	170 ± 20	25

MICs ± SEM (n=3), values are rounded to the nearest 10 ppm ae.

Killing curves were constructed for environments with only antibiotics or a combination of antibiotic and herbicide. Four patterns were observed, depending on the specific antibiotic and herbicide combination. (1) Increases and (2) decreases in the MIC of an antibiotic. (3) No statistically significant effect. (4) Statistical significance was achieved without any change in MIC.

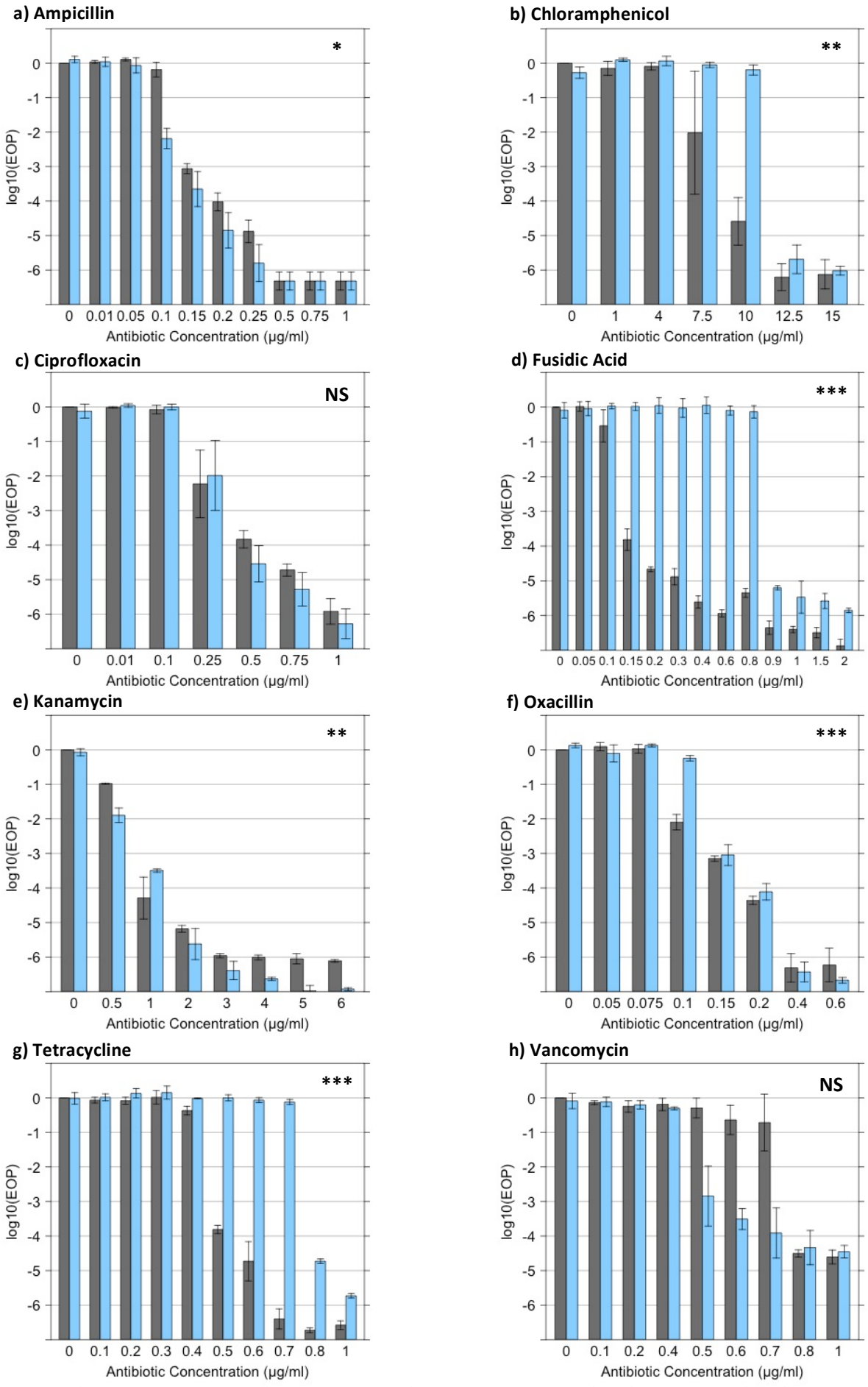
2,4-D

The herbicide 2,4-D induced statistically significant responses to six of the eight antibiotics tested (Figure 2.2). The two antibiotics where no significant effect was observed were vancomycin ($p = 0.188$) and ciprofloxacin ($p = 0.932$). 2,4-D caused a 1.3-fold decrease in the MIC of vancomycin although overall the herbicide did not cause a significant change in EOP. 2,4-D caused a decrease in antibiotic tolerance for two of the six significant combinations, ampicillin ($p = 0.015$) and kanamycin ($p = 0.008$). The combination of 2,4-D and the β-lactam ampicillin resulted in a 1.3-fold decrease in MIC compared to the antibiotic only treatment whilst 2,4-D and the aminoglycoside kanamycin had a significant interaction but this did not result in a change in MIC (Table 2.2).

S. aureus exhibited a statistically significant increase in antibiotic tolerance exposed to 2,4-D and chloramphenicol ($p = 0.003$), fusidic acid ($p = 2.00 \times 10^{-16}$), oxacillin ($p = 9.71 \times 10^{-4}$), and tetracycline ($p = 2.00 \times 10^{-16}$). This resulted in fold-changes in MIC that varied from no

change for oxacillin, a 1.3-fold increase for chloramphenicol, and a 1.6-fold increase for tetracycline (Table 2.2). The largest effect of all combinations was a 6-fold increase in MIC of fusidic acid (Table 2.2). This corresponds to a change in MIC from 0.15 μg/mL to 0.9 μg/mL of fusidic acid (Figure 2.2).

Figure 2.2 – Survival of *S. aureus* on a range of concentrations of antibiotics with and without 2,4-D.



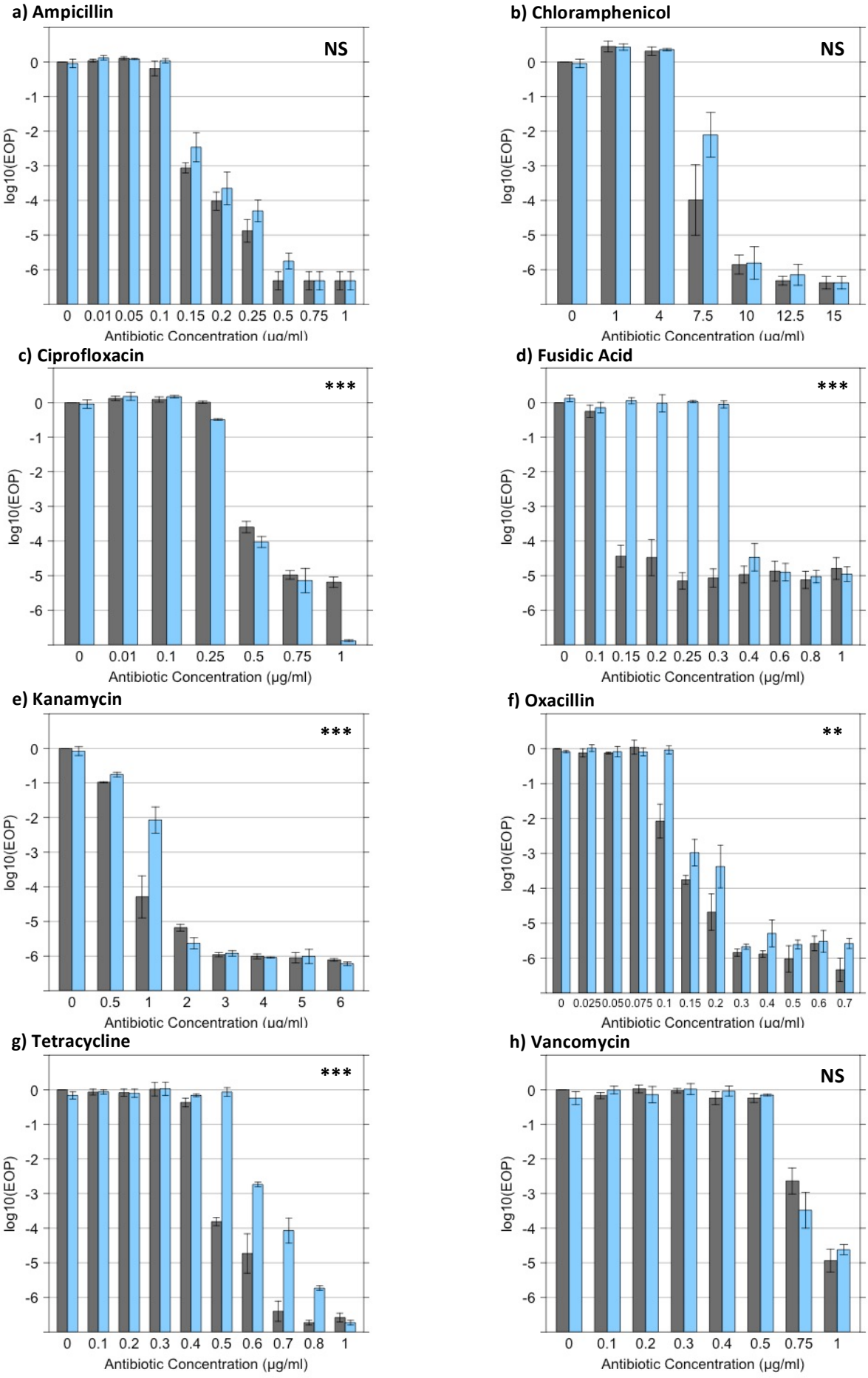
Survival of *S. aureus* on a range of concentrations of eight antibiotics with (blue) and without (grey) 2,4-D. Survival is reported as log-transformed EOP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Kamba

Kamba caused statistically significant changes to the antibiotic tolerance of five of the eight tested antibiotics (Figure 2.3). The three unaffected antibiotics were ampicillin ($p = 0.767$), chloramphenicol ($p = 0.18$), and vancomycin ($p = 0.228$).

Ciprofloxacin ($p = 3.59 \times 10^{-5}$) was the only antibiotic tested where a decrease in tolerance was observed upon addition of the herbicide, however this did not result in a change in MIC. *S. aureus* tolerated a greater concentration of fusidic acid ($p < 2.00 \times 10^{-16}$), kanamycin ($p = 8.56 \times 10^{-6}$), oxacillin ($p = 0.006$), and tetracycline ($p = 1.13 \times 10^{-13}$) when Kamba was present. Kamba induced a 1.4-fold change in MIC for tetracycline, 2-fold changes in MIC for kanamycin and oxacillin, and a 2.7-fold change in MIC for fusidic acid (Table 2.2).

Figure 2.3 – Survival of *S. aureus* on a range of concentrations of antibiotics with and without Kamba.



Survival of *S. aureus* on a range of concentrations of eight antibiotics with (blue) and without (grey) Kamba. Survival is reported as log-transformed EOP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Roundup

Roundup induced statistically significant responses in six of the eight antibiotics tested (Figure 2.4). Ciprofloxacin ($p = 0.772$) and chloramphenicol ($p = 0.958$) were the two antibiotics where the addition of Roundup had no statistically significant effect on growth and there were no changes in MIC (Table 2.2).

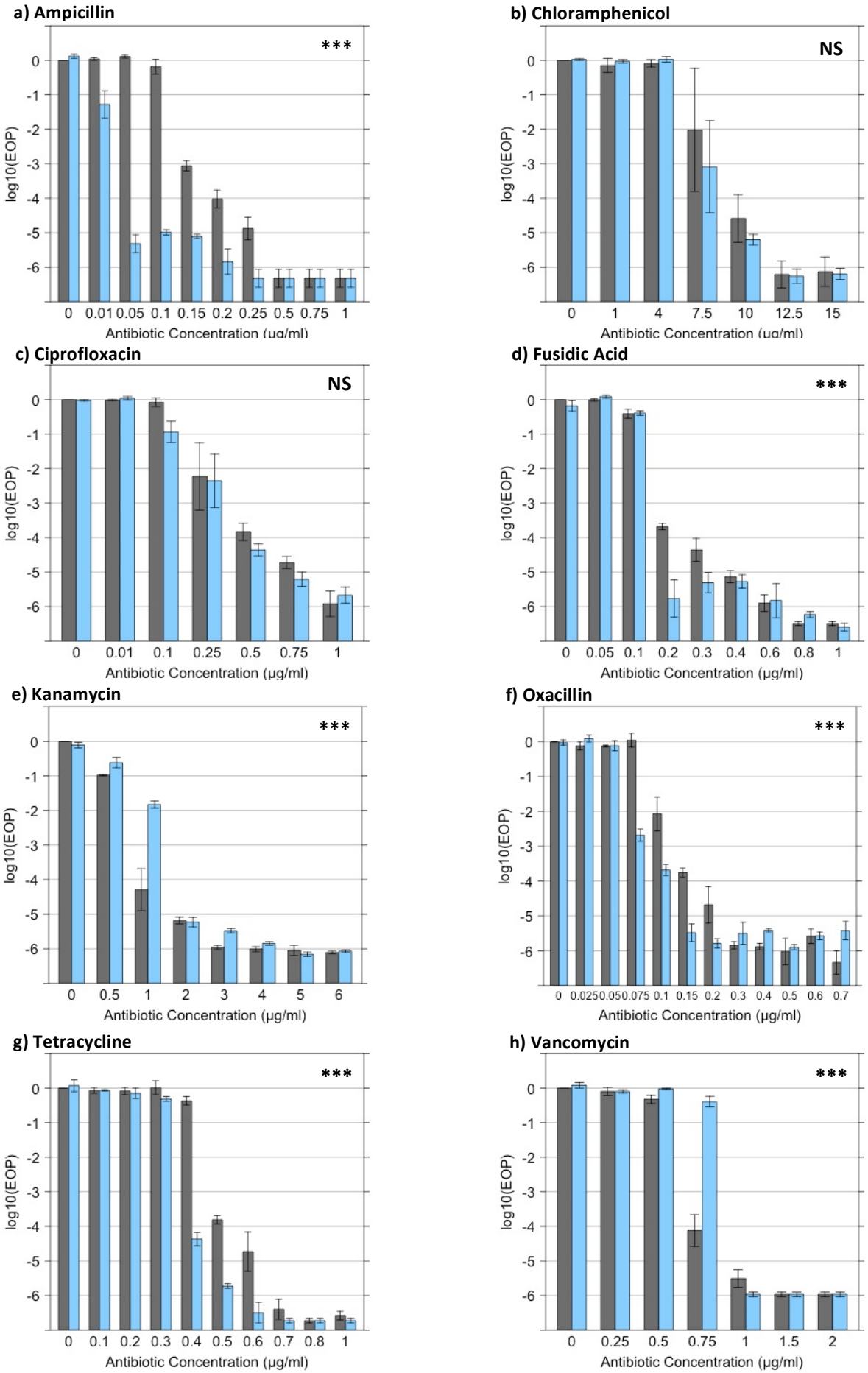
Roundup increased *S. aureus* tolerance to two antibiotics, kanamycin ($p = 1.63 \times 10^{-7}$) and vancomycin ($p = 2.24 \times 10^{-12}$). This corresponded to a 2-fold and 1.3-fold increase in MIC, respectively. There were statistically significant interaction terms for the following antibiotics upon addition of Roundup: ampicillin ($p = 2.00 \times 10^{-16}$), fusidic acid ($p = 1.60 \times 10^{-4}$), oxacillin ($p = 4.59 \times 10^{-10}$), and tetracycline ($p = 1.23 \times 10^{-13}$). The MIC was reduced for these antibiotics when Roundup was present. The decreases in MIC were 4-fold for ampicillin, 1.5-fold for oxacillin, 1.3-fold for tetracycline and no change in MIC for fusidic acid (Table 2.2).

Table 2.2 – Fold-change in antibiotic concentration necessary to cause a 1000-fold reduction in EOP.

	Amp	**Cm**	**Cip**	**Fus**	**Kan**	**Oxa**	**Tet**	**Vanc**
2,4-D	**1.3**	1.3	NS	6	0	0	1.6	**1.3** (NS)
Kamba	NS	NS	0	2.7	2.0	2.0	1.4	NS
Roundup	**4**	NS	NS	0	2	**1.5**	**1.3**	1.3

Grey shading indicates the herbicide caused an increase in antibiotic tolerance. Bold indicates the herbicide caused a decrease in antibiotic tolerance. NS: herbicide by antibiotic interaction term was not significant, 0: The herbicide by antibiotic interaction term was significant but the 1000-fold reduction in EOP occurs at the same antibiotic concentration for antibiotic only and antibiotic + herbicide treatments. Herbicide concentrations were 914 ppm ae for 2,4-D; 1371 ppm ae for Kamba; and 25 ppm ae for Roundup.

Figure 2.4 – Survival of *S. aureus* on a range of concentrations of antibiotics with and without Roundup.



Survival of *S. aureus* on a range of concentrations of eight antibiotics with (blue) and without (grey) Roundup. Survival is reported as log-transformed EOP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

2.3.2 Minimum herbicide concentrations necessary to induce changes in the antibiotic tolerance of S. aureus

In certain combinations commercial herbicide formulations changed the antibiotic tolerance of *S. aureus*. In the next series of experiments, I determined the herbicide concentrations necessary to induce these effects. Only combinations of herbicide and antibiotic that had a statistically significant interaction term were used in the dose response assay. The minimum herbicide concentration that caused a 100-fold change in EOP relative to the antibiotic control was chosen as the endpoint in the dose response assay (Table 2.3). The antibiotic concentration at which the herbicide caused the maximum difference in EOP was used in each assay (Table 2.3).

Table 2.3 – Minimum herbicide concentrations required to induce a 100-fold change in EOP.

	Amp	**Cm**	**Cip**	**Fus**	**Kan**	**Oxa**	**Tet**	**Vanc**
2,4-D	914 (0.1)	50 (10)	–	300 (0.4)	NC (0.5)	NC (0.1)	300 (0.5)	–
Kamba	–	–	NC (1)	300 (0.25)	NC (1)	NC (0.05)	1371 (0.5)	–
Roundup	10 (0.075)	–	–	25 (0.2)	25 (1)	15 (0.075)	10 (0.4)	20 (0.75)

Herbicide concentrations are given in ppm ae. (–) indicates the herbicide and antibiotic combination had a non-significant interaction term and no dose response assay was performed. Certain combinations had significant killing curves but did not reach the 100-fold threshold as indicated by the no change (NC). Antibiotic concentrations used are shown in parentheses (µg/mL).

Of the three herbicides, Roundup induced changes in antibiotic response of *S. aureus* at the lowest concentrations (Table 2.3). This is not unexpected as both the MIC of Roundup and the concentration used in the killing curve assays is far lower than the respective values for Kamba and 2,4-D (Table 2.1). There were several combinations of herbicide and antibiotic that only caused small changes in EOP that did not reach the 100-fold change in EOP

threshold. This can mainly be attributed to small differences in EOP with and without the herbicide in the killing curve assay, which were nevertheless sufficient to give a significant p-value. The 100-fold change in EOP threshold was chosen as an arbitrary limit, changes in EOP below this threshold could still be of biological importance.

2,4-D

The lowest 2,4-D concentration necessary to cause a 100-fold change in EOP was 50 ppm ae when coupled with chloramphenicol ($p = 0.012$). The other antibiotics required higher herbicide concentrations of 300 ppm ae for fusidic acid ($p = 0.016$) and tetracycline ($p = 0.011$), while ampicillin ($p = 0.019$) required 914 ppm ae, the highest concentration tested. The two other antibiotics, kanamycin ($p = 0.057$) and oxacillin (p 0.017) did not cause a 100-fold change in EOP even at the highest herbicide concentration tested (Figure 2.5).

Kamba

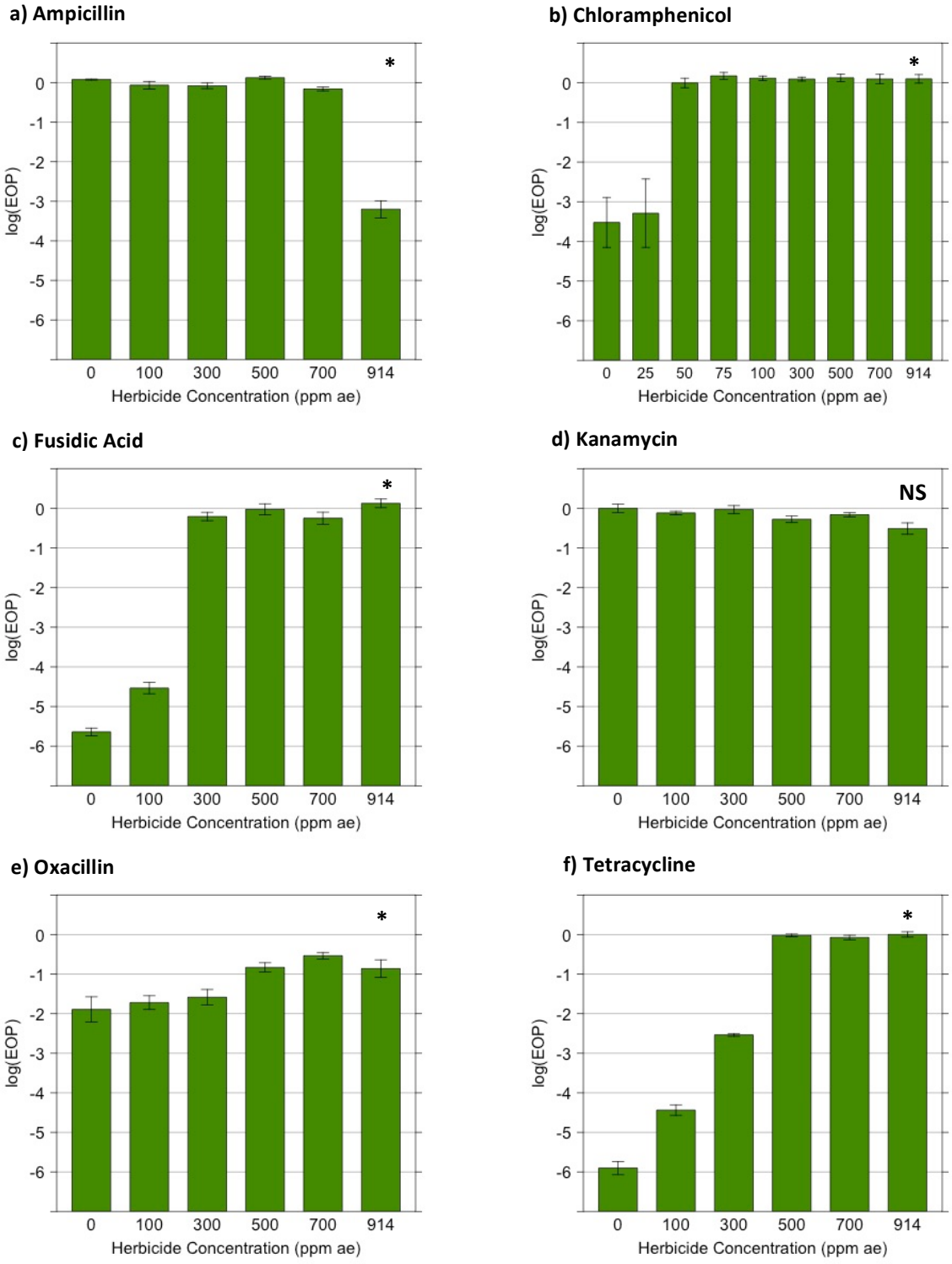
Five antibiotics were tested to determine the minimum Kamba concentration necessary to cause a 100-fold change in EOP. Three antibiotics did not reach this threshold, ciprofloxacin ($p = 0.944$), kanamycin ($p = 0.165$), and oxacillin ($p = 0.035$). Of the remaining two antibiotics, fusidic acid ($p = 0.020$) required 300 ppm ae Kamba for a 100-fold change in EOP to be observed whilst tetracycline ($p = 0.021$) required the maximum concentration tested of 1371 ppm ae (Figure 2.6).

Roundup

The lowest Roundup concentration needed to induce 100-fold change in EOP was 10 ppm ae for ampicillin ($p = 0.021$) and tetracycline ($p = 0.023$). A higher concentration of 15 ppm

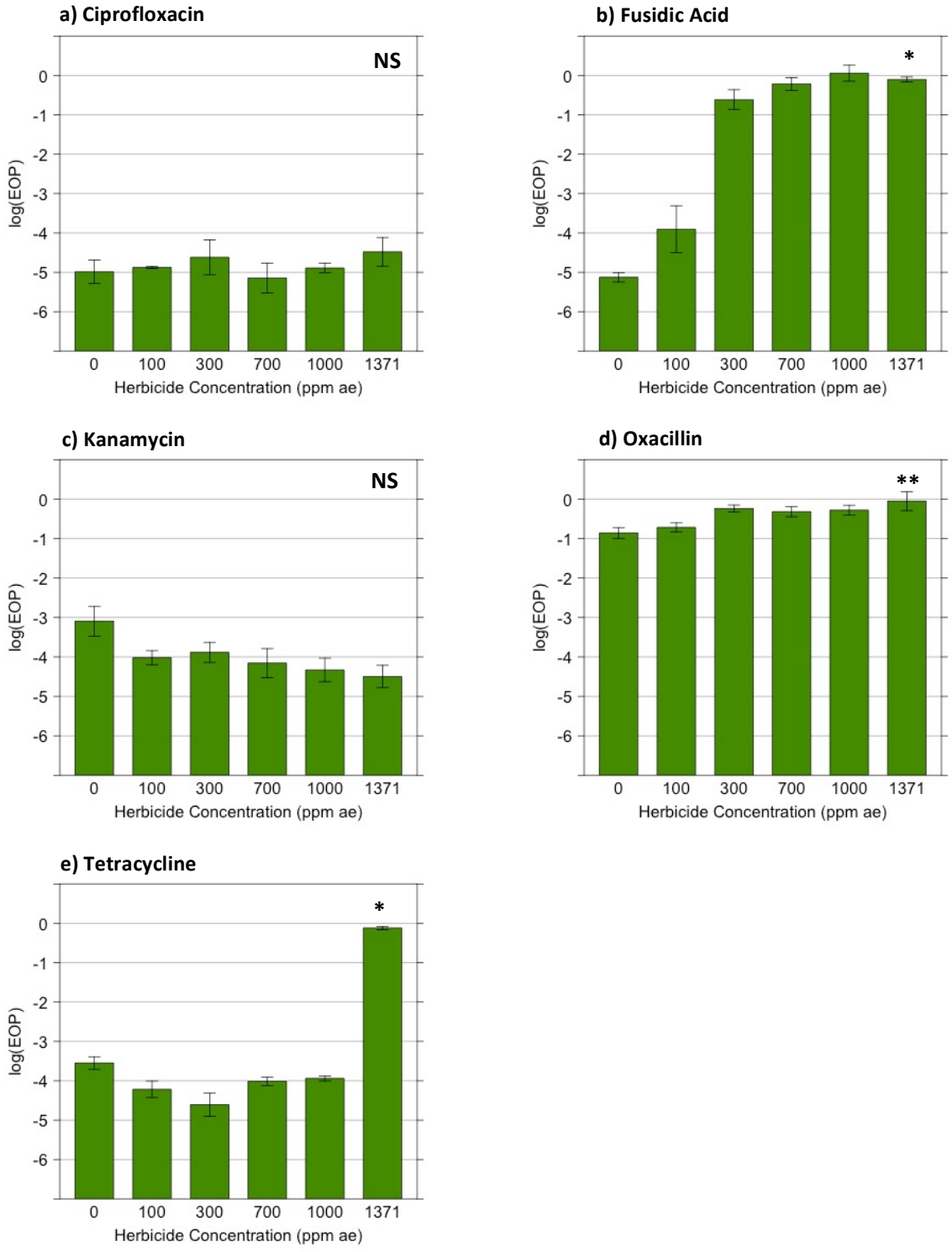
ae was required for oxacillin (p = 0.003), 20 ppm ae for vancomycin (p = 0.017), and 25 ppm ae for fusidic acid (p = 0.030) and kanamycin (p = 0.023) (Figure 2.7).

Figure 2.5 – Dose response curves of *S. aureus* in the presence of 2,4-D.



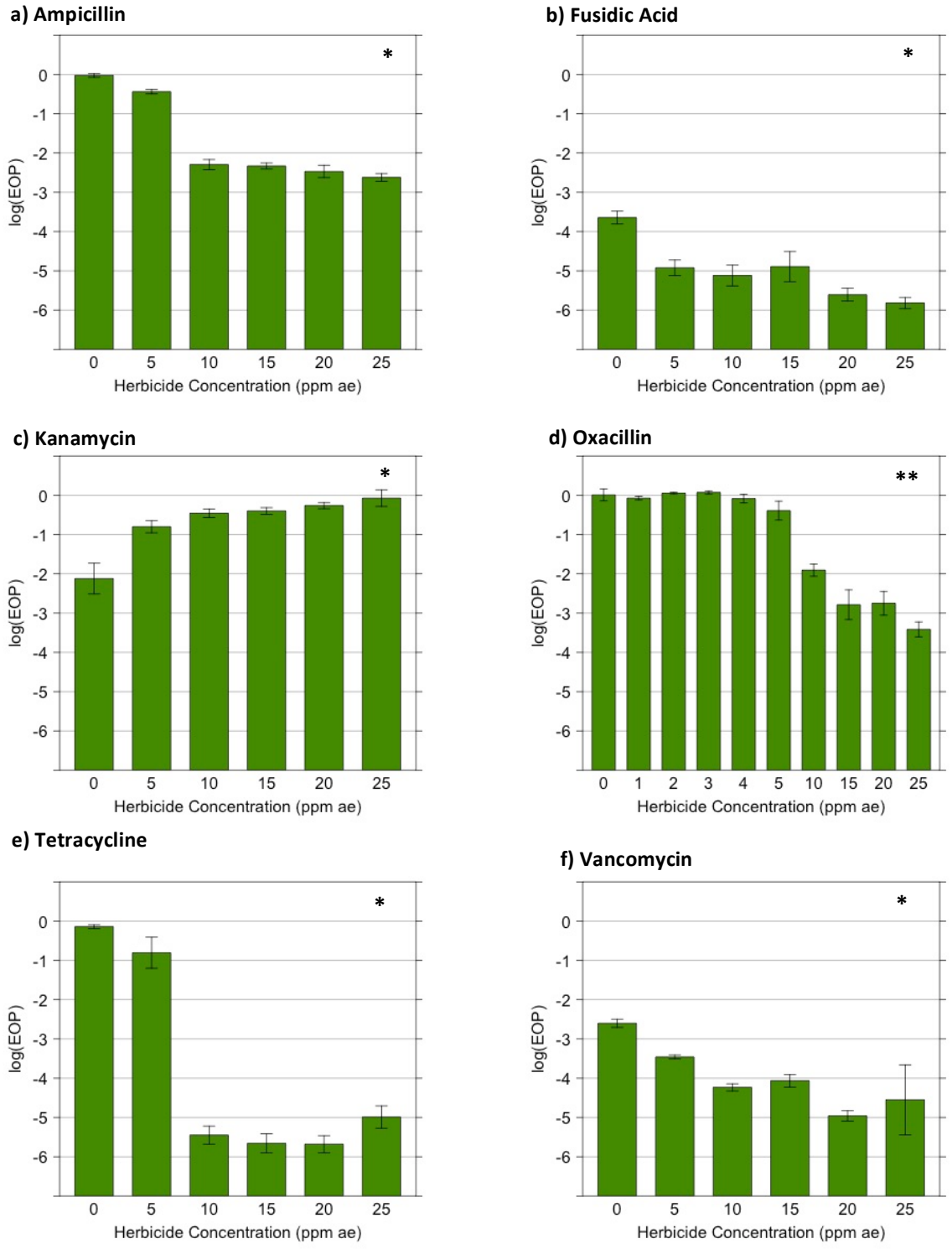
Survival of *S. aureus* on various antiboitics in the presence of a range of 2,4-D concentrations. Survival is reported as the log-transformed EOP scores ± SEM (n=3) . These curves were used to determine the minimum herbicide concentration required to induce a 100-fold change in EOP compared to the antibiotic only control (Table 2.3).

Figure 2.6 – Dose response curves of *S. aureus* in the presence of Kamba.



Survival of *S. aureus* on various antiboitics in the presence of a range of Kamba concentrations. Survival is reported as the log-transformed EOP scores ± SEM (n=3) . These curves were used to determine the minimum herbicide concentration required to induce a 100-fold change in EOP compared to the antibiotic only control (Table 2.3).

Figure 2.7 – Dose response curves of *S. aureus* in the presence of Roundup.



Survival of *S. aureus* on various antiboitics in the presence of a range of Roundup concentrations. Survival is reported as the log-transformed EOP scores ± SEM (n=3) . These curves were used to determine the minimum herbicide concentration required to induce a 100-fold change in EOP compared to the antibiotic only control (Table 2.3).

2.4 Discussion

Herbicides are commonly used chemicals that have been detected in wide range of environments. The use of herbicides is continuing to expand in certain areas including the USA where there was a 239 million kg increase in herbicide use from 1996 to 2011 (Benbrook, 2012). It is necessary to understand any unintended effects of the herbicides, in this instance on bacteria and their tolerance to antibiotics. This chapter provides further evidence that herbicides can alter the antibiotic tolerance of bacteria, in particular *Staphylococcus aureus.*

2.4.1 Commercial herbicide formulations can cause changes in the antibiotic tolerance of S. aureus

Previous work has shown that commercial herbicide formulations and their associated active ingredients can cause changes in the antibiotic responses of *E. coli* and *S. enterica* (Kurenbach *et al.*, manuscript in preparation; Kurenbach *et al.*, 2015). The key aim of the work described in this chapter was to determine if this effect could be extended to a different type of bacteria, the pathogenic Gram-positive bacteria represented by *S. aureus.* Three herbicide formulations were tested for their ability to induce changes in the tolerance of *S. aureus* to eight antibiotics (Table 2.2). 2,4-D caused no change in MIC, increases in MIC that ranged from 1.3-fold (chloramphenicol) to 6-fold (fusidic acid), and decreases in MIC of 1.3-fold (ampicillin and vancomycin). Kamba caused either no change in MIC or an increase in MIC that ranged in magnitude from 1.4-fold (tetracycline) to 2.7-fold (fusidic acid). Roundup caused no change in MIC, increases in antibiotic MIC ranging from 1.3-fold

(vancomycin) to 2-fold (kanamycin), and decreases in the MIC that ranged from 1.3-fold (tetracycline) to 4-fold (ampicillin).

Previous work has determined the changes in MIC for *E. coli* and *S. enterica* using the same herbicide formulations and five of the antibiotics tested in this study (ampicillin, chloramphenicol, ciprofloxacin, kanamycin and tetracycline) (Kurenbach *et al.*, 2015). Although it is difficult to make direct comparisons, as the herbicide concentrations used were different, some broad trends can be observed. Roundup in particular was highly toxic to *S. aureus* with an MIC of just 170 ppm ae compared to 7,400 ppm ae for the *E. coli* strain previously tested and 6190 ppm ae for the *S. enterica* strain (Kurenbach *et al.*, 2015). The mechanism through which Roundup kills *S. aureus* is unclear. Glyphosate kills plants through inhibition of the EPSPS enzyme, however the purified *S. aureus* EPSPS enzyme exhibited a high level of tolerance to glyphosate (Priestman *et al.*, 2005). This was indicative of a class II EPSPS enzyme, a version of which is used in to make crops glyphosate-tolerant (Priestman *et al.*, 2005). It would be interesting to compare the MIC of glyphosate alone to the MIC of Roundup as this could provide evidence that it is not the active ingredient that is responsible for the Roundup toxicity or that glyphosate has another target in *S. aureus*. The components of Roundup other than the active ingredient are not known so it is difficult to identify what other chemicals may be responsible.

In general, the size of the fold-change in MIC was similar to that observed by Kurenbach *et al.* (2015), ranging from no change to approximately 6-fold in both studies. However if data specific to fusidic acid, which was not used in the previous study, is removed, then the fold-changes in MIC only range from no change to 2-fold making the magnitude of change in *S. aureus* smaller overall than those observed for *E. coli* and *S. enterica*. Nevertheless, even

small changes in MIC can affect the success of antibiotic therapy. For instance, increased vancomycin tolerance has been significantly associated with *S. aureus* bacteraemia treatment failure, even when the MIC remains in the susceptible range (Britt *et al.*, 2017; Wang *et al.*, 2006). Roundup increased the MIC of vancomycin from 0.75 µg/mL to 1 µg/mL; this level of increased resistance could cause an increased likelihood of treatment failure. Sakoulas *et al.* (2004) found that vancomycin was only 9.5% effective in the treatment of MRSA bacteraemia when the isolate had a vancomycin MIC of 1 to 2 µg/mL.

Certain trends were also observed by Kurenbach *et al.* (2015). For instance they observed that regardless of the herbicide or bacteria, tolerance to ciprofloxacin always increased. This was not the case for *S. aureus,* which showed no major changes upon ciprofloxacin exposure with non-significant responses to 2,4-D and Roundup and a very small, though significant, decrease in EOP in the presence of Kamba that did not change the MIC. One proposed mechanism through which the herbicides cause an increase in antibiotic tolerance is through the activation of efflux pumps (Kurenbach *et al.*, 2015). The components of the AcrAB-TolC efflux pump were found to play at least a partial role in the herbicide induced adaptive resistance (Kurenbach *et al.*, manuscript in preparation). The AcrAB-TolC efflux pump is in the RND superfamily, which is only found in Gram-negative bacteria (Sun *et al.*, 2014). Differences in the efflux pumps present in *S. aureus* and *E. coli* could explain why certain patterns of adaptive resistance no longer hold for the Gram-positive *S. aureus*. In general, the herbicide active ingredients induce the changes in EOP in the same direction as the commercial formulation in *S. enterica* (Gibson, 2016). At this stage, no work has been performed to determine if this holds true for *S. aureus*.

2.4.2 Concentrations of herbicides within potential exposure levels for bacteria are sufficient to induce a change in antibiotic response

Governments and regulatory agencies decide the maximum residue limits (MRLs) of herbicide active ingredients that are acceptable in various food groups (Table 2.4). These decisions are often based on guidance by international bodies such as the Codex Alimentarius Commission, which maintains a collection of standards, guidelines and recommendations relating to food safety. This includes descriptions of the maximum allowable residues of herbicides on commodities grown for human and animal consumption, broken down by the commodity and herbicide (Codex Alimentarius Commision, 2016). These standards apply mainly to food products that are traded internationally. The standards are also based on active ingredient concentration and do not take into account any other chemicals within a herbicide formulation. The minimum concentration of herbicide necessary to induce a change in the antibiotic tolerance of *S. aureus* was compared to the internationally recognised guidelines for herbicide MRLs from the Codex Alimentarius.

The minimum inducing concentrations for 2,4-D and Kamba were above those that are allowable as residues in human food products (Table 2.4). However, all of the Roundup concentrations found to cause a change in the antibiotic tolerance of *S. aureus* were below the MRLs for glyphosate. The highest maximum residue limit is 30ppm ae, which applies to cereal grains and rape seed, and is below the highest Roundup concentration used in these experiments. Roundup induced changes in antibiotic tolerance at 10 ppm ae for tetracycline and ampicillin, 15 ppm ae for oxacillin, 20 ppm ae for vancomycin and 25 ppm ae for fusidic acid and kanamycin. Sugar beet, dry soya bean, unprocessed wheat bran and

sugar cane molasses all have MRLs that are 10 ppm ae or more (Codex Alimentarius Commision, 2016). The MRLs for animal feed are within the concentrations observed to induce changes in antibiotic tolerance upon 2,4-D exposure. A minimum 2,4-D concentration of 50 ppm ae was required to cause a change in ampicillin tolerance while 300 ppm ae was needed to cause changes in fusidic acid and tetracycline tolerance. These are below the MRL for hay, and 50 ppm ae is below the MRL for wheat straw. The concentrations of Kamba necessary to cause a change in antibiotic tolerance are all above the MRL for animal feed as well as human food products. MRLs only apply to animal feed that is internationally traded, if the feed is used domestically, higher herbicide levels may be acceptable.

Table 2.4 – Summary of Maximum Residue Limits for each herbicide active ingredient.

Active Ingredient	Human Food Products	Animal Feed
2,4-d (2,4-D)	0.01 – 5	10 – 400
Dicamba (Kamba)	0.01 – 10	0.06 – 50
Glyphosate (Roundup)	0.05 – 30	50 – 500

Range of MRLs for human food products and animal feed from the Codex Alimentarius Commission (Codex Alimentarius Commision, 2016). Commercial herbicide formulations are given in brackets following the active ingredient, all concentrations are in ppm ae.

Maximum residue limits are set for the final food product that will be sold or traded (Horváth *et al.*, 2014). However, the herbicide application rate recommended by manufacturers is far higher than this in order to halt the growth of pests and prevent the evolution of herbicide resistant weeds (Tharp & Kells, 1999). Therefore, the amount of herbicide applied to crops can be far higher than the residues in the final product as the herbicide will break down or be diluted over time. The recommended application rates of the herbicide formulations used in this study are all above the minimum concentrations necessary to induce a change in the antibiotic tolerance of *S. aureus* (Table 2.5).

Table 2.5 – Herbicide Recommended Application Rates.

Herbicide	Recommended Application Rate
2,4-D	33,080
Kamba	415 – 2,200
Roundup	2,664 – 87,912

Recommended application rates for three commercial herbicide formulations, 2,4-D Amine 800 WSG (Agpro, Auckland, NZ), Kamba^{500} (Nufarm, Otahuhu, NZ), and Roundup Weed Killer (Monsanto, Australia). Concentrations are given in ppm ae.

S. aureus is an important human and animal pathogen that is becoming increasingly resistant to antibiotics (Centers for Disease Control and Prevention, 2013). Approximately 20% of people have persistent colonisation of *S. aureus* in the nasal passages while up to 60% of people are intermittent carriers (Kluytmans *et al.*, 1997). While *S. aureus* is most commonly found in the nose it can also colonise the skin and cause a range of diseases including cellulitis, urinary tract infections and sepsis (Wertheim *et al.*, 2005). *S. aureus* is also commonly found in livestock and higher rates of MRSA colonisation has been observed in swine farmers (Wardyn *et al.*, 2015). Furthermore, application of swine manure onto crops has also been shown to increase rates of community-associated MRSA infections (Casey *et al.*, 2013). It is necessary to understand the effects of chemicals such as herbicides on the antibiotic tolerance of *S. aureus* in order to prevent further development of resistance. The observation that 2,4-D and Kamba cause increases in fusidic acid tolerance in *S. aureus* is particularly relevant to New Zealand where high rates of fusidic acid resistance are already observed (Williamson *et al.*, 2014).

Herbicide-induced changes in antibiotic tolerance have now been described for three species of bacteria: *E. coli, S. enterica,* and *S. aureus*. It is likely that this effect will extend beyond these species. Bacteria in a number of environments may be exposed to herbicides and as such may exhibit these changes in antibiotic tolerance if co-exposed to antibiotics.

In addition to herbicide treated plants, both soils and water have been found to contain herbicide active ingredients (Battaglin *et al.*, 2014; Kremer & Means, 2009). Antibiotics have also been found in waterways and soils due to the use of animal manure as a fertiliser (Kemper, 2008; Mackie *et al.*, 2006). Farm animals and bees are also treated prophylactically with antibiotics to prevent infections (Dolliver & Gupta, 2008; Shea, 2003; Tian *et al.*, 2012). Antibiotics are sprayed on plants directly in some cases, for instance streptomycin is used in the treatment of *Pseudomonas syringae* pv. actinidiae (psa) infected kiwifruit crops (Frampton *et al.*, 2014). Other potential exposure pathways include bacteria that are found on or in humans, animals and insects. Humans and animals can come into contact with herbicides via residues on food, inhalation and skin contact caused by spray drift or direct herbicide usage. Glyphosate (found in Roundup) residues, for example, have been found in honey (Rubio *et al.*, 2015). Salicylic acid and Kamba can have additive effects, increasing the survival of *S. enterica* in the presence of chloramphenicol at concentrations that separately cannot improve survival to the same degree (Gibson, 2016). The potential for other combinations of herbicides to have additive effects has not yet been tested however, it is possible that low concentrations of a number of chemicals could be sufficient to induce a change in antibiotic tolerance.

Both herbicides have also been detected in farm runoff and aquatic environments near farmlands (Kuo *et al.*, 2012). 2,4-d and dicamba were detected in 100% and 86% of samples in a survey of drinking water reservoirs in North America respectively, although the concentrations did not exceed drinking water guidelines (Donald *et al.*, 2007). However, both herbicides were detected in Canadian rainfall at concentrations that occasionally exceeded the Canadian Drinking Water Guidelines in samples taken in Alberta during

agricultural seasons (Filkowski *et al.*, 2003). A study of surface waters in California found 5 or more different pesticides in over 50% of samples with 2,4-d and dicamba two of the most commonly detected herbicides (Ensminger *et al.*, 2013). Herbicide residues have even been detected in carpet dust within homes (Colt *et al.*, 2004). Dicamba and 2,4-d were among the most commonly detected herbicides at concentrations up to 0.037 ppm and 1.5 ppm respectively (Colt *et al.*, 2004).

There are many potential exposure pathways through which species of bacteria including, but not limited to, *S. aureus, E. coli,* and *S. enterica* can come into contact with low concentrations of herbicides and antibiotics. The combination of these chemicals, among others, has been shown to induce changes in antibiotic tolerance. These changes need to be understood and considered when determining how herbicides and antibiotics can be safely used and preserved in the future.

Chapter Three

Evolution of acquired resistance during herbicide-induced increases in antibiotic tolerance

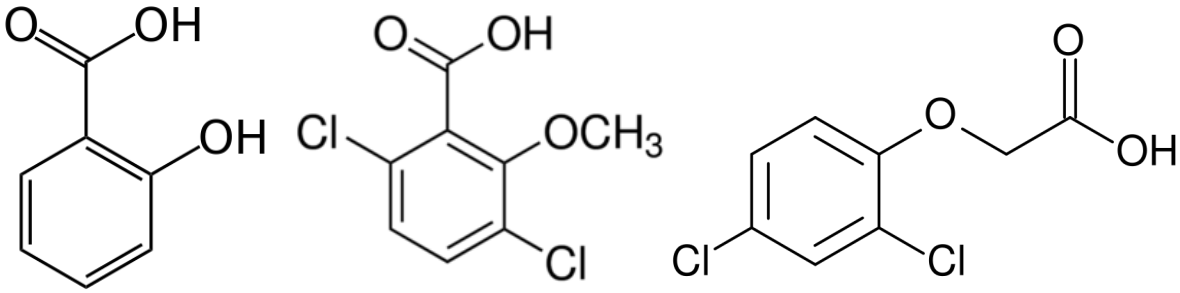
3.1 Introduction

Herbicides can induce adaptive changes in the antibiotic tolerance of *Salmonella enterica* and *Escherichia coli* (Kurenbach *et al.*, 2015). Depending on the specific combination of organism, antibiotic, and herbicide, one of three responses was observed: 1) increase, 2) decrease, and 3) no change in antibiotic tolerance. These changes were phenotypically reversible; bacteria reverted to their original tolerance level after removal of herbicides (Kurenbach *et al.*, 2015). I hypothesised that this adaptive resistance could, over time, lead to an increased frequency of acquired antibiotic resistance that would persist even in the absence of the herbicide.

Adaptive antibiotic resistance caused by exposure to salicylates has been shown to cause an increased frequency of acquired resistance. Salicylates were first shown to cause adaptive resistance to a range of antibiotics (chloramphenicol, tetracycline, ampicillin, and nalidixic acid) by Rosner (1985). This phenomenon has since been observed in a number of different species. Salicylate activates the *E. coli mar* operon causing down-regulation of OmpF synthesis, and up-regulation of AcrAB-TolC synthesis, decreasing influx and increasing efflux from the cell (Alekshun & Levy, 1999; Cohen *et al.*, 1989; Cohen *et al.*, 1988).

Salicylates were shown to increase the frequency of ciprofloxacin resistance in populations of both *Staphylococcus aureus* (Gustafson *et al.*, 1999) and *Campylobacter jejuni* (Shen et al., 2011). The CmeABC efflux system of *C. jejuni* was found to be a key factor affecting the frequency of ciprofloxacin resistant mutants (Shen et al., 2011). Point mutations in *gyrA* are enough to increase *C. jejuni* resistance to ciprofloxacin and other fluoroquinolones; however, this phenotype cannot be maintained without CmeABC at high antibiotic concentrations (Shen *et al.*, 2011; Yan *et al.*, 2006). The CmeABC efflux pump belongs to the RND superfamily, as does AcrAB-TolC, which has been implicated in herbicide-induced adaptive resistance in *E. coli* (Kurenbach *et al.*, manuscript in preparation; Shen *et al.*, 2011). In addition, the chemical structure of salicylate is very similar to the active ingredients of two of the herbicides studied, 2,4-d and dicamba (Figure 3.1).

Figure 3.1 – Chemical structures of salicylate, dicamba, and 2,4-d.



Salicylate Dicamba 2,4-d

It is worth investigating if herbicides that cause adaptive resistance can also cause an increased frequency of acquired resistance. I performed a series of experiments to determine if the exposure of *S. enterica* to Kamba or Roundup with ciprofloxacin could lead to an increased frequency of acquired resistance. Ciprofloxacin was chosen because it was the only antibiotic in the initial study to which all herbicides increased tolerance (Kurenbach *et al.*, 2015), and increased frequencies of ciprofloxacin resistance had been found following salicylate exposure (Gustafson *et al.*, 1999; Shen *et al.*, 2011). Fluoroquinolones such as

ciprofloxacin are used to treat a wide range of infections in humans and animals (Kemper, 2008). It is important to understand how bacteria can become resistant to these drugs and how environmental contaminants may contribute to the development of resistance.

3.2 Methods

3.2.1 Bacterial strain, culture conditions and chemicals

Salmonella enterica serovar Typhimurium strain SL3770 (LT2, *pyr*$^{+}$, *rfa*$^{+}$) was used for all experiments (Roantree *et al.*, 1977). This strain was also used in the original work by Kurenbach *et al.* (2015). All strains were stored and grown as described in Section 2.2.1. The chemicals used in these assays were the antibiotic ciprofloxacin (cip) and commercial herbicides Kamba and Roundup.

3.2.2 Determination of minimum inhibitory concentrations

The MIC of ciprofloxacin was determined in LB broth medium and on LB agar. The MIC of ciprofloxacin when exposed to each herbicide was determined in liquid LB medium only. To determine the MIC in broth, 10 mL cultures in LB medium were started with 100 µL of a saturated culture of *S. enterica* (OD_{600} ≈ 1), with each containing a different ciprofloxacin concentration. These were incubated for approximately 24 hours at 37°C with rotation providing aeration. The cfu/mL of the initial saturated culture was determined by inoculating LB agar plates with 10 µL droplets of *S. enterica* at various dilutions representing 10^{-2} to 10^{-8} of the original culture. The same method was used to calculate the cfu/mL for each antibiotic concentration tested. Plates were incubated for 16 hours at 37°C before colonies were counted. The MIC in broth was the lowest concentration of ciprofloxacin or

ciprofloxacin and herbicide that showed no increase in cfu/mL following 24 hours incubation at 37°C.

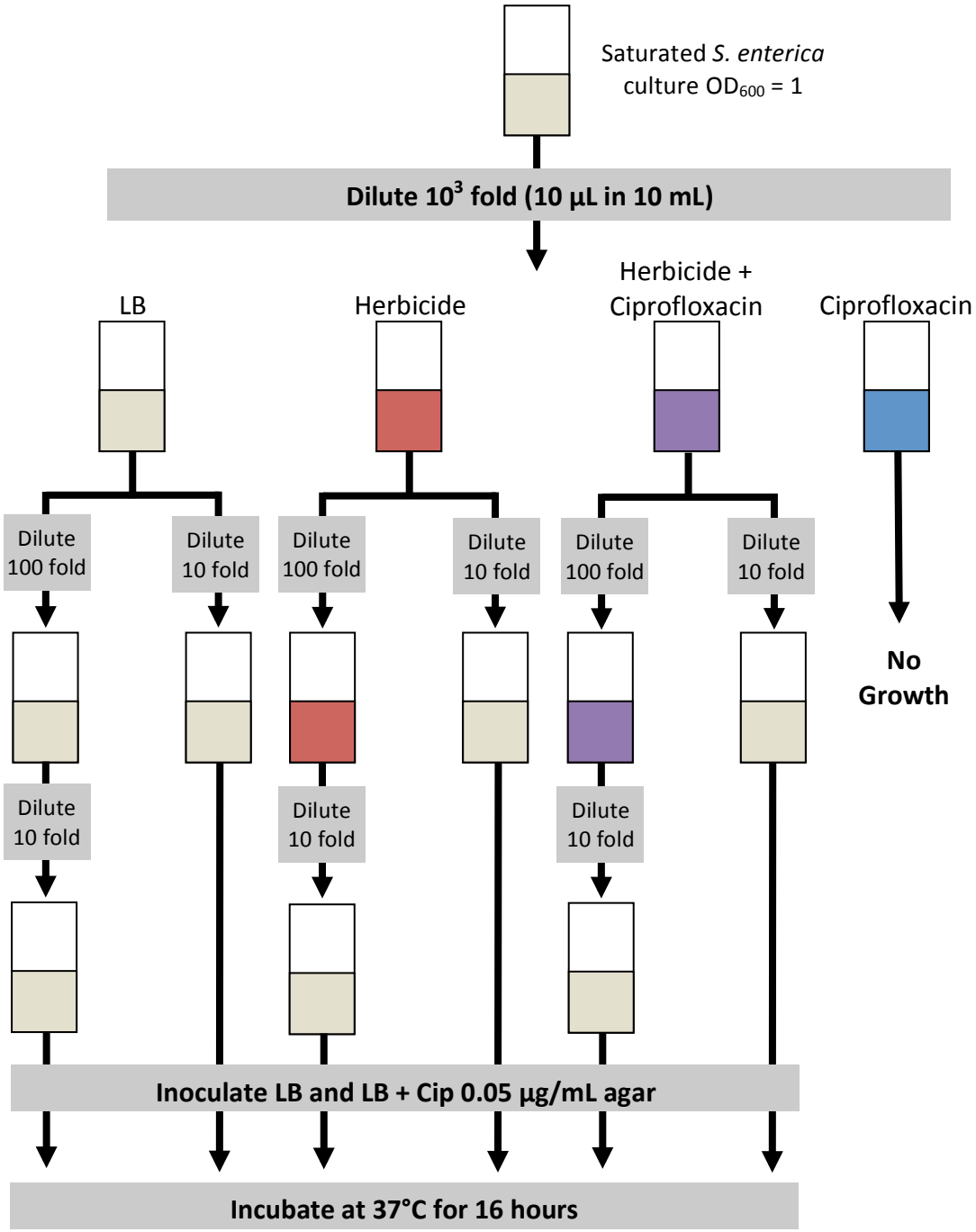
LB agar supplemented with increasing concentrations of ciprofloxacin was inoculated with 10 µL droplets of a saturated culture of *S. enterica* at various dilutions ranging from 10^2 to 10^8 (a 'spot plate' as described in Section 2.2.2). The MIC was defined as the ciprofloxacin concentration that prevented the growth of any colonies on the 10^3 dilution.

3.2.3 Determining the effect of herbicide and ciprofloxacin combinations on the frequency of resistant bacteria

Four culture treatments were used: LB, herbicide only, ciprofloxacin only and ciprofloxacin + herbicide (Figure 3.2). 10 mL of LB supplemented as appropriate for the treatment, were inoculated with 10 µL of a saturated culture of *S. enterica* and incubated at 37°C with rotation providing aeration for 24 hours. After 24 hours a 100-fold dilution was made into 10 mL of fresh LB medium under the same treatment conditions, and a 10-fold dilution was made into 10 mL of LB only. These cultures were incubated at 37°C for 24 hours with rotation providing aeration. A 10-fold dilution into LB was then made for each culture still in a treatment. These cultures were incubated at 37°C for 24 hours with rotation providing aeration. The purpose of the 10-fold dilution in LB and the subsequent 24 hour culturing period was to allow the culture to physiologically revert from the adapted phenotype induced by the herbicide. The titre of every culture was calculated by inoculating LB agar plates with 10 µL droplets of *S. enterica* at various dilutions ranging from 10^2 to 10^8. LB + ciprofloxacin agar plates were inoculated at the same dilutions from all cultures started by a 10-fold dilution in fresh LB. Additional LB + ciprofloxacin plates were inoculated with 100 µl of each culture, giving a 10^1 dilution, lowering the detection limit. Plates were incubated for

approximately 16 hours at 37°C before colonies were counted. When this experiment was first performed, the antibiotic only control was not included and the 10^3-fold dilution was a 100-fold dilution. For experiments that used the low ciprofloxacin concentration the ciprofloxacin only treatment was treated the same as the other three treatments.

Figure 3.2 – Schematic diagram of the protocol to test the effect of herbicides on the frequency of ciprofloxacin resistance.



S. enterica was grown in LB (beige) supplemented with herbicides (red), ciprofloxacin (blue) or both (purple). Each culture was grown for 24 hours at 37°C with aeration provided by rotation. A titre was calculated for every culture following incubation. The herbicides used were Kamba (1827 ppm ae) and Roundup (1245 ppm ae). Ciprofloxacin concentration was either 0.01 µg/mL or 0.07 µg/mL.

The titre of each culture on LB was used to estimate the number of generations that had occurred during each 24 hour culturing period (Figure 3.3). At the end of each experiment, the resistance frequency was calculated for every treatment after 24 and 48 hours of exposure to the treatment conditions (Figure 3.4).

$$Number\ of\ Generations = \frac{log(\frac{Final\ Titre}{Initial\ Titre})}{log(2)}$$

Figure 3.3 – Equation to calculate the number of generations.

$$Resistance\ Frequency = \frac{Titre\ of\ bacteria\ on\ cip}{Titre\ of\ bacteria\ on\ LB}$$

Figure 3.4 – Equation to calculate the frequency of resistant mutants within a population.

To test how frequently the starting culture of *S. enterica* contained a pre-existing resistant mutant 10 replicates of 10 mL of LB medium inoculated with a single colony were incubated at 37°C with aeration to $OD_{600} \approx 1$. Cultures were diluted 100-fold in 10 mL fresh LB medium containing 0.07 µg/mL ciprofloxacin and incubated for 24 hours at 37°C with rotation providing aeration. A titre on LB agar was determined for each saturated culture as previously described, 100 µL of each culture was also used to inoculate LB agar containing 0.05 µg/mL ciprofloxacin. Each LB + ciprofloxacin culture was checked for growth following incubation; if cultures were cloudy growth had occurred.

3.2.4 Measuring mutagenicity of Roundup

Twelve independent cultures of *S. enterica* were inoculated from single colonies mixed in 10 mL LB medium, 6 of which also contained 1245 ppm ae Roundup. Cultures were incubated at 37°C with rotation providing aeration to $OD_{600} \approx 1$. LB agar plates supplemented with 100

µg/mL rifampicin (Sigma-Aldrich, USA) were inoculated with a 10^1 and 10^2-fold dilution of each culture. A titre on LB agar was also determined for each saturated culture as previously described. Plates were incubated at 37°C for 16 hours before colonies were counted. The frequency of rifampicin resistant mutants was then compared between LB and LB + Roundup treatments. This assay was adapted from Miller *et al.* (1999).

3.2.5 Statistical analysis

R was used for all statistical analyses (R Core Team, 2015). For the resistance frequency experiments, an ANOVA was used to analyse the randomised complete block design. A log transformation of the resistance frequency data was carried out after testing the assumptions of normality and equal variance. Tukey's HSD test was used to determine which treatments were significantly different from each other. For this analysis, residual plots were used to test for normality and equality of variance.

For the rifampicin experiment, a two-tailed t-test was used to determine if there was any significant difference in the frequency of rifampicin resistant mutants between the LB and LB + Roundup treatments. All graphs were created in R using the lattice package (Sarkar, 2008).

3.3 Results

3.3.1 Minimum Inhibitory Concentrations

The MIC of ciprofloxacin was determined in both liquid broth and on solid media, while only MICs in broth were determined for ciprofloxacin + Kamba and ciprofloxacin + Roundup (Table 3.1). On solid LB medium, 0.05 µg/mL ciprofloxacin was used to select for

ciprofloxacin resistant mutants. In LB broth 0.07 ciprofloxacin µg/mL was used, a concentration above the MIC of ciprofloxacin for the *S. enterica* strain used in isolation but below MIC when either herbicide was also present.

Table 3.1 – Minimum inhibitory concentrations of ciprofloxacin on *S. enterica.*

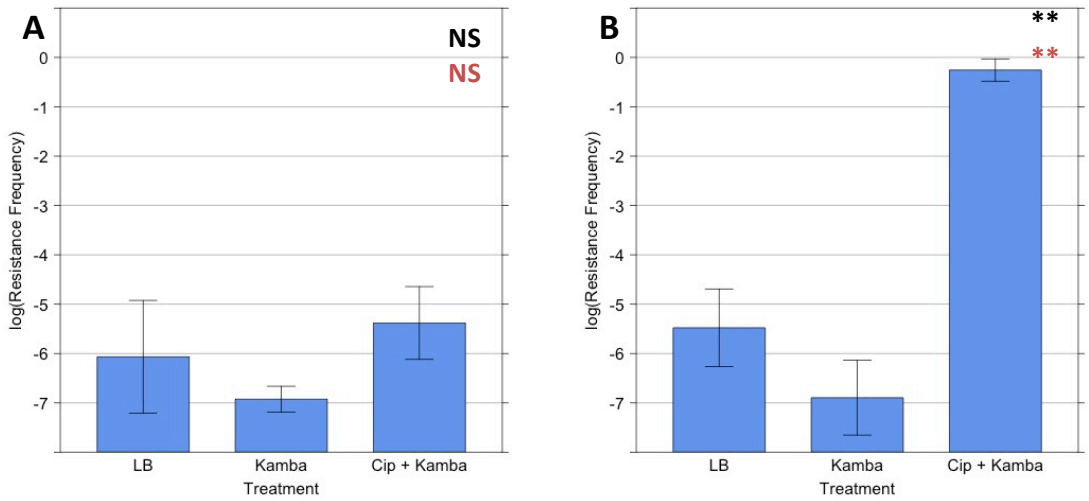
	Cip	**Cip**	**Cip + Kamba**	**Cip + Roundup**
Medium	Solid	Broth	Broth	Broth
MIC (µg/mL)	0.035 ± 0.00	0.053 ± 0.006	0.11 ± 0.01	0.20 ± 0.05

MIC values are the average of three independent experiments ± SEM (n=3). Herbicide concentrations were 1827 ppm ae Kamba and 1245 ppm ae Roundup.

3.3.2 Preliminary resistance frequency experiments

S. enterica was exposed to three treatments: LB control, herbicide only, and herbicide + ciprofloxacin. The ciprofloxacin only treatment was included only in later experiments. This experiment was performed for two antibiotic and herbicide combinations: ciprofloxacin + Kamba and ciprofloxacin + Roundup. *S. enterica* was exposed to treatments for approximately 6 and 12 generations before culturing in liquid LB medium under non-selective conditions to allow for any herbicide-induced adaptive changes to subside. Resistance frequencies were determined for each treatment after 24 and 48 hours of exposure to each treatment.

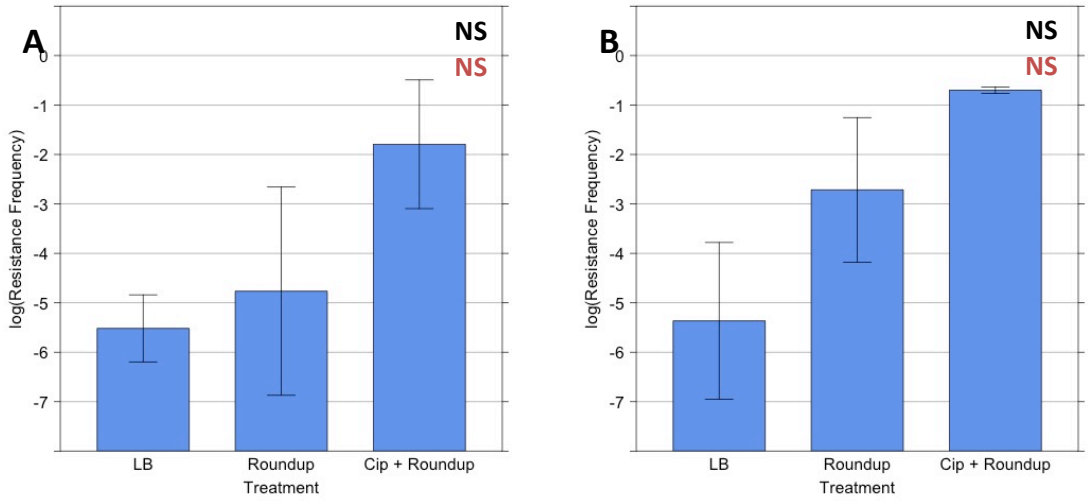
Figure 3.5 – Frequency of ciprofloxacin-resistant *S. enterica* following Kamba exposure.



Resistance frequencies following (A) 24 or (B) 48 hours in each treatment. Values are reported as log-transformed frequencies ± SEM (n=3). Asterisks show the results of the comparison between LB (black) or Kamba (red) and the combination treatment, and correspond to the significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

For the ciprofloxacin and Kamba combination, no significant differences were observed in the frequency of resistance between any treatments after one 24 hour culturing period (Figure 3.5). However, after 48 hours of exposure to the treatment, the frequency of ciprofloxacin resistance was significantly higher in the ciprofloxacin + Kamba treatment than the Kamba only ($p = 0.004$) or LB treatments ($p = 0.009$). There was no significant difference between the LB and Kamba treatments ($p = 0.346$). The increase in antibiotic tolerance caused by Kamba can provide the opportunity for rare resistant mutants in the population to arise, compete with the susceptible genotype, and increase in proportion within the population.

Figure 3.6 – Frequency of ciprofloxacin-resistant *S. enterica* following Roundup exposure.



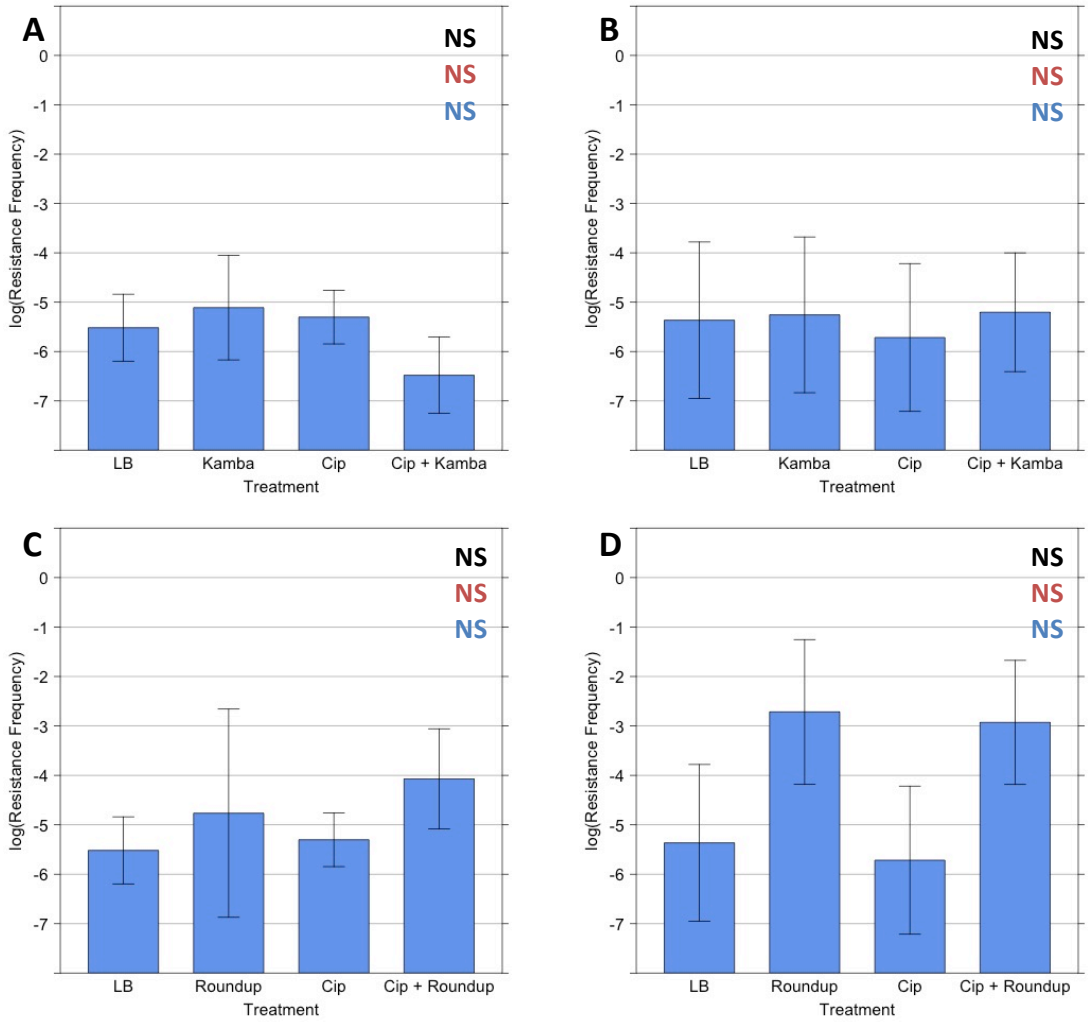
Resistance frequencies following (A) 24 or (B) 48 hours in each treatment. Values are reported as log-transformed frequencies ± SEM (n=3). Asterisks show the results of the comparison between LB (black) or Roundup (red) and the combination treatment, and correspond to the significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

There were no significant differences observed between any of the treatments in the ciprofloxacin and Roundup tests for either the 24 or 48 hour culturing periods (Figure 3.6). This is partly due to the high degree of variability between replicates, as illustrated by the large error bars (Figure 3.6). A greater number of replicates would be required to determine if the small changes in resistance frequency between treatments were significant.

Taking into account that the presence of Kamba led to a higher frequency of ciprofloxacin resistant bacteria, I endeavoured to test whether these effects could still be seen in an environment under much weaker selection (i.e. very low ciprofloxacin concentrations) (Figure 3.7). Roundup and a low ciprofloxacin concentration was also tested as there was a trend of a higher frequency of ciprofloxacin resistant bacteria when a higher concentration was used, even though it was not statistically significant. The ciprofloxacin concentration

was reduced 7-fold to 0.01 µg/mL for these experiments, below the MIC of ciprofloxacin alone (Table 3.1).

Figure 3.7 – Frequency of ciprofloxacin resistant *S. enterica* following exposure to sub-lethal ciprofloxacin concentrations.



Resistance frequencies following 24 (A & C) or 48 (B & D) hours in each treatment. A & B contained 1831 ppm ae Kamba, C & D used 1245 ppm ae Roundup. Resistance frequency values are reported as log-transformed frequencies ± SEM (n=3). Asterisks show the results of the comparison between LB (black), Herbicide (red), or cip (blue) with the cip + antibiotic treatment, and correspond to the significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

There were no significant differences observed between treatments at a low ciprofloxacin level (Figure 3.7). The Tukey's HSD test confirmed that there were no significant differences between any of the treatments from the 24 hour or 48 hour exposure groups. The same result was observed for both Kamba and Roundup. It is not surprising that no

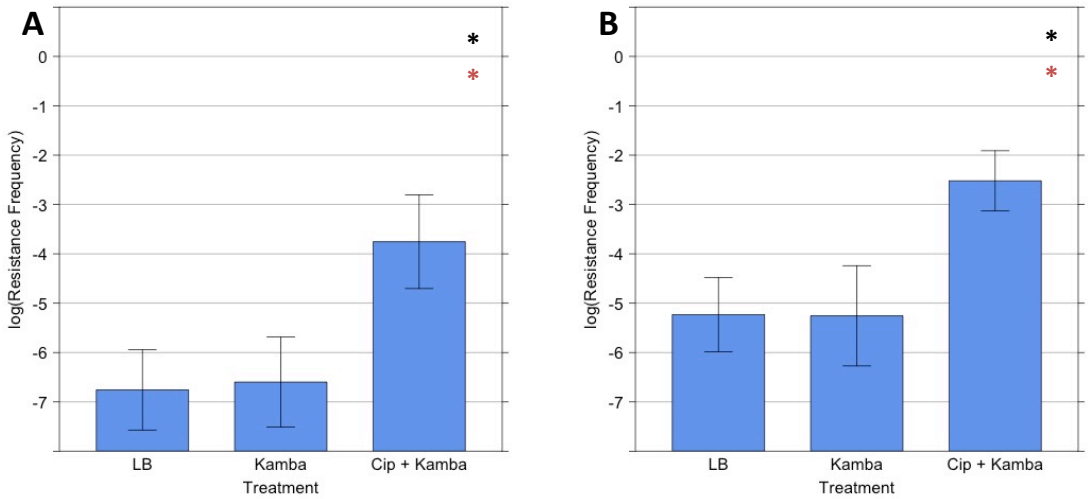
significant effect was observed for Roundup as the higher ciprofloxacin concentration also did not cause any significant differences in the frequency of ciprofloxacin resistance. While it does appear that the Roundup only and ciprofloxacin + Roundup treated cultures have a higher frequency of resistant mutants than cultures in the other treatments, this was not statistically significant and the variation between replicates was too high to draw any conclusions. The large error bars, the result of high variation between replicates, made it difficult to accurately assess if one treatment was significantly different from the others. Approximately 12 generations occurred in each treatment, similar to the number of generations in the higher ciprofloxacin conditions. However, it is possible that because selection is not as strong at a lower antibiotic concentration, the experiment would need to be extended for a greater number of generations for any difference in resistance frequency across treatments to be accurately detected. Alternatively, the concentration of ciprofloxacin was too low to provide any selective advantage to the rare mutants that arose under these conditions.

To test whether the high degree of variation observed in the experiments above was due to a small number of resistant bacteria that had arisen randomly in the starting culture of *S. enterica*, 10 cultures of *S. enterica* were exposed to 0.07 µg/mL ciprofloxacin grown for 24 hours. Three of the 10 cultures were turbid following incubation indicating they were inoculated with a pre-existing mutant. These rare bacteria were able to reproduce in concentrations of ciprofloxacin above the wild-type MIC. This provides an explanation as to why there was a high degree of variation between replicates in the control culture conditions.

3.3.3 The effect of herbicides on the evolution of ciprofloxacin resistance

To account for this underlying level of resistant bacteria within the population, the stringency of the experimental setup was increased by a) increasing the initial dilution step from 1:100 to 1:1000, and b) adding an additional ciprofloxacin only control. The experiment was abandoned and restarted using a fresh culture if growth occurred in the antibiotic only culture. Growth in ciprofloxacin was evidence of pre-existing resistant bacteria in the source culture. Experiments were repeated six times.

Figure 3.8 – Frequency of ciprofloxacin-resistant *S. enterica* following Kamba exposure from cultures free of pre-existing mutants.

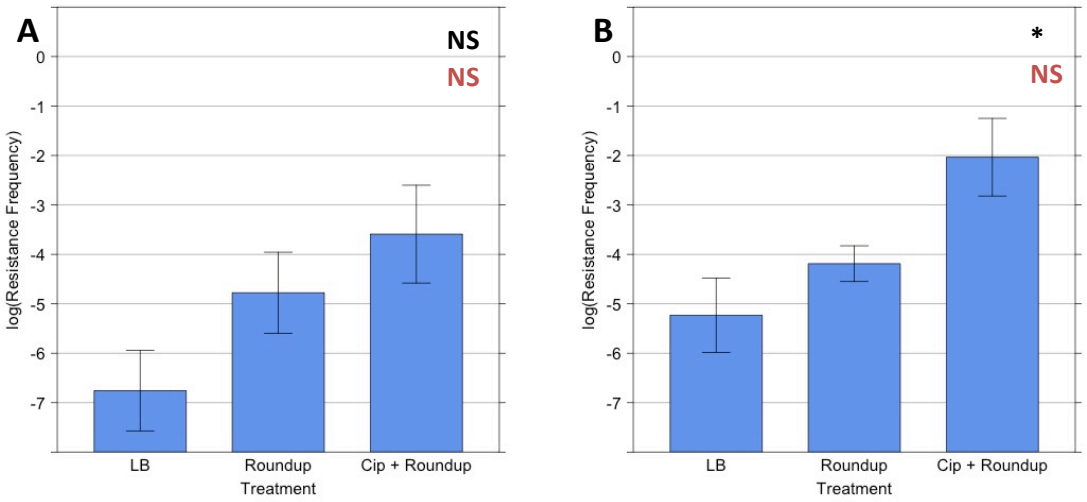


Resistance frequencies following (A) 24 or (B) 48 hours in each treatment. Values are reported as log-transformed frequencies ± SEM (n=6). Asterisks show the results of the comparison between LB (black) or Kamba (red) and the combination treatment, and correspond to the significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

The ciprofloxacin and Kamba treatment had a significantly higher frequency of resistant bacteria than the LB and Kamba treatments for both the 24 and 48 hour incubation periods (Figure 3.8). The increased dilution factor at the start of each experiment may help to explain why there was a significant difference after 24 hours whereas in the previous experiments 48 hours were needed to observe the effects. The number of generations that occurred in 24 hours was similar to the number of generations that occurred in 48 hours in

the initial experiments, because the lower number of starting cells allows for more generations of growth. The difference in the frequency of resistant bacteria between treatments is not as pronounced in these repeated experiments as it was in the original set of experiments. This may be the result of different antibiotic stocks being used because there was a time gap between experiments. Alternatively, the ciprofloxacin and Kamba treatment might have been artificially high due to 'jackpot' (Luria & Delbrück, 1943) mutants in the first set of experiments.

Figure 3.9 – Frequency of ciprofloxacin-resistant *S. enterica* following Roundup exposure from cultures free of pre-existing mutants.



Resistance frequencies following (A) 24 or (B) 48 hours in each treatment. Values are reported as log-transformed frequencies ± SEM (n=6). Asterisks show the results of the comparison between LB (black) or Roundup (red) and the combination treatment, and correspond to the significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

In the ciprofloxacin and Roundup combination, 48 hours of culturing was necessary to observe a significant change in the frequency of resistant mutants (Figure 3.9). There were no significant differences between treatments following one 24 hour culturing step. After 48 hours, the ciprofloxacin and Roundup treatment had a significantly higher frequency of resistant bacteria than LB treatment ($p = 0.017$). The Roundup only treatment had no

significant difference in resistant bacteria when compared to the ciprofloxacin + Roundup treatment ($p = 0.102$). However, Roundup alone was not sufficient to cause a significant increase in mutation frequency, as there was no significant difference between the Roundup and LB treatments ($p = 0.528$). The presence of ciprofloxacin and Roundup significantly increased the frequency of resistant bacteria only when compared to the LB control. However, the resistance frequency of the ciprofloxacin and Roundup treatment was still approximately 100-fold higher than the Roundup treatment indicating that although this result may not be statistically significant, it could have some biological relevance. The improved experimental design allowed this increase to be detected where it was not clear in earlier experiments.

The different treatment cultures had different growth rates; to account for this the resistance frequency per generation was also calculated. However, this did not change the conclusions (data not shown). Both Kamba and Roundup caused an increase in the frequency of resistant mutants when compared to a non-selective environment, the LB treatment. At concentrations that would otherwise be lethal to *S. enterica*, these herbicides induced an adaptive resistance to ciprofloxacin, which allowed for the development and proliferation of acquired resistance within a population. Neither herbicide in isolation caused a significant change in resistance frequency when compared to LB.

3.3.4 The effect of Roundup on mutation rate

There was a high variation in the frequency of ciprofloxacin resistant mutants in treatments containing Roundup (Figure 3.9). *S. enterica* was incubated in liquid LB medium or LB + 1245 ppm ae Roundup until the cultures reached saturation. The frequency of rifampicin resistant mutants was then measured for both treatments. Rifampicin resistance is caused

by a single mutation in *rpoB*, making it a convenient system to observe changes in mutation rates. There was no significant difference in the frequency of rifampicin resistant bacteria in the cultures exposed to 10mM Roundup compared to the bacteria only exposed to LB ($p = 0.3873$). It would appear that Roundup is not a mutagen for *S. enterica*.

3.4 Discussion

Herbicides can cause adaptive resistance to a range of antibiotics (Kurenbach *et al.*, 2015). This chapter provides evidence that this adaptive resistance can lead to acquired resistance where bacteria are no longer dependent on herbicide induction to survive at an antibiotic concentration that was previously lethal.

3.4.1 Herbicides cause an increased frequency of acquired resistance

The frequency of ciprofloxacin resistance was measured following culturing in LB, or when supplemented with Kamba or Roundup only, and each herbicide with ciprofloxacin. *S. enterica* was exposed to each treatment for 24 or 48 hours before the resistance frequency was measured. The maximum number of generations ranged from 10 to 18, as different treatments had different growth rates. The resistance frequency per generation was also calculated to account for this, however it had no effect on the final results and their statistical significance (data not shown).

When *S. enterica* was exposed to ciprofloxacin and Kamba the frequency of resistant bacteria was approximately 100-fold higher than the frequency observed for the Kamba and the LB only treatment after 48 hours of treatment exposure. The ciprofloxacin and Kamba treatment had a resistance frequency that was significantly different to the Kamba and LB controls, which were not significantly different from each other. When *S. enterica* was

exposed to ciprofloxacin and Roundup the frequency of resistant bacteria was 100-fold higher than the Roundup treatment and 1,000-fold higher than the LB treatment after 48 hours of exposure to the treatments. Although the ciprofloxacin and Roundup resistance frequency only differed significantly from the LB treatment and not the Roundup treatment this result still has biological relevance. Roundup alone caused no significant increase in the frequency of ciprofloxacin mutants and did not increase the frequency of rifampicin resistance in the mutagen test.

These experiments have also been performed using *E. coli* and those have returned similar results for the ciprofloxacin and Roundup combination (B. Kurenbach, personal communication). In contrast to *S. enterica*, the ciprofloxacin and Kamba combination did not cause a significant change in the frequency of resistant *E. coli* (B. Kurenbach, personal communication).

When bacteria are exposed to sub-lethal herbicide concentrations adaptive resistance is induced, which may enable bacteria to survive antibiotic concentrations that would otherwise be lethal. This increase in survival provides an opportunity for acquired resistance to develop, in this case in the form of a mutation (Kurenbach *et al.*, 2015). I would hypothesise that there is a fitness cost to herbicide-induced adaptive resistance, but that this cost is minor compared to the fitness cost of the antibiotic. The reason ciprofloxacin resistant mutants are found at such a high frequency in the ciprofloxacin and herbicide treatments is that the ciprofloxacin resistance mutations provide a fitness advantage, perhaps by also making the mutants independent of the costs of adaptive resistance, allowing the mutants to increase in proportion in the population. The presence of the herbicide allows for heterogeneity within the population to increase and competition

between traits can occur with the fittest bacteria surviving. Mutants isolated from each treatment could be tested as described in Section 2.2.2 to determine how any mutations have affected the herbicide-induced adaptive response. For instance, these mutants may be less response to the herbicide or they may be hyper efflux mutants, pumping out more herbicide and ciprofloxacin and thus achieving higher fitness by one mechanism.

As previously discussed, herbicides and antibiotics have been detected in a wide range of environments where bacteria could be exposed to both chemicals. Antibiotics have been found in waterways and soils due to the use of animal manure as a fertilizer (Kemper, 2008; Mackie *et al.*, 2006). Similarly, herbicide active ingredients have been detected in soil and waterways (Battaglin *et al.*, 2014; Kremer & Means, 2009). The herbicide concentrations used in these experiments are above the MRLs for human food and animal feed but are below application rates (Table 2.4 & 2.5). These experiments only used one herbicide concentration but it is possible that lower concentrations could cause a similar effect. The dose response assay in Chapter 2 showed that lower herbicide concentrations could still induce a change in antibiotic tolerance. Concentrations of Roundup and Kamba as low as 248 and 183 ppm ae, respectively, caused adaptive resistance to ciprofloxacin in *S. enterica* in the study by Kurenbach *et al.* (2015). The magnitude of the change in survival drops as the herbicide concentration drops so it possible that at lower herbicide concentrations the effects observed in this chapter would not be as strong. However, I hypothesise that the herbicides could still cause an increased frequency of acquired resistance at lower herbicide concentrations.

The ciprofloxacin concentration used to select for bacteria with acquired resistance was only slightly above MIC. There a number of mechanisms through which bacteria can

achieve differing levels of ciprofloxacin resistance including mutations in genes encoding the antibiotic target such as *gyrA, gyrB, parC* and *parE* (Aldred et al., 2014). These genes encode the two subunits of gyrase (GyrA and GyrB) and topoisomerase IV (ParC and ParE) (Aldred et al., 2014). Low levels of ciprofloxacin resistance can be achieved through increased expression of efflux pumps, most often caused by mutations in regulatory proteins (Aldred et al., 2014).

A number of ciprofloxacin resistant mutants from each treatment were isolated and frozen for long-term storage. It would be interesting to further characterise these bacteria, including determining the MIC of ciprofloxacin. Whole genome sequencing could be used as an unbiased search for mutations that have occurred in each isolate. Of particular interest would be if there were any patterns in the mechanisms through which resistance occurred based on treatment. Any mutations in antibiotic target genes would represent a more serious public health risk than the low-level resistance mechanisms (Aldred *et al.*, 2014; Poole, 2007). However, even small increases in MIC have been associated with increased treatment failures (Britt *et al.*, 2017; Chisholm *et al.*, 2010). In addition, low level resistance mutations can improve survival and create a favourable background for the development of other high-level antibiotic resistance mechanisms (Aldred *et al.*, 2014; Fernández *et al.*, 2011).

A wide range of compounds can cause adaptive resistance phenotypes. Some of these compounds, such as salicylate and now herbicides have already been shown to cause increased frequencies of acquired resistance (Gustafson *et al.*, 1999; Shen *et al.*, 2011). The shift from adaptive to acquired resistance is important to understand as it represents a serious potential threat to human and animal health.

Chapter Four

Selection in favour of acquired resistance following herbicide-induced decreases in antibiotic tolerance

4.1 Introduction

Herbicides cause physiologically reversible decreases in the tolerance of *E. coli* to certain antibiotics (Kurenbach *et al.*, 2015). While this decrease in tolerance initially appears beneficial for humans, as lower antibiotic concentrations are able to inhibit bacterial growth, it was hypothesised that it could increase selective pressure in favour of more resistant bacteria within a population (Kurenbach *et al.*, 2015). Recent evidence suggests that selection in favour of resistant bacteria in a heterogeneous population can occur at antibiotic concentrations far below the MIC of either the resistant or susceptible strain (Andersson & Hughes, 2012; Gullberg *et al.*, 2011). These studies have shown that sub-lethal concentrations of antibiotics can increase the overall resistance levels of bacterial populations.

I have performed a series of experiments to determine if the addition of a herbicide that reduces the tolerance of bacteria to an antibiotic would be sufficient to cause selection in favour of resistant bacteria at an antibiotic concentration that in isolation had no selective effect. If this were indeed the case, it would increase the range of environments where low-levels of antibiotics could cause selection in favour of resistance, as other chemicals that are also present in the environment might have to be considered. For instance, animal manure

can contain antibiotics and antibiotic resistant bacteria, which may then be exposed to herbicides if spread on soils before crops are grown (Dolliver & Gupta, 2008).

4.2 Methods

4.2.1 Bacterial strains, culture conditions and chemicals

The strains and plasmids used in this chapter are outlined in Table 4.1. All strains used in this study were stored and grown as described in Section 2.2.1. All chemicals used are as described in Section 2.2.1. The additional antibiotics used for these experiments were streptomycin sulfate salt and nalidixic acid sodium salt, both purchased from Sigma-Aldrich (USA). When selecting for resistant strains the antibiotic concentrations used were as follows: chloramphenicol (Cm) 20 µg/mL, nalidixic acid (Nx) 60µg/mL, ampicillin (Amp) 100µg/mL, tetracycline (Tet) 15µg/mL, and streptomycin (Str) 50µg/mL.

Table 4.1 – *E. coli* strains and plasmids used in this study.

Bacteria	**Description**	**Reference**
LU1	*leuB6, pyrF, rpsL20* (Str^r)	Laboratory strain
AH205	LU1 pBR322 (Tet^r, Amp^r)	This study
AH206	LU1 Jp103 (partial Tet^r)	This study
AH209	LU1 Nx^r (spontaneous), Tet^s	This study
SB21	*hsdS, leuB6, thr*	Heinemann and Sprague Jr (1989)
AH201	SB21 pBR322 (Tet^r, Amp^r)	This study
AH214	SB21 pAH14 (Cm^r), Tet^s	This study
AH211	SB21 pAH11 (Cm^r), Str^s	This study
JB436	SB21 Nx^r (spontaneous)	Heinemann *et al.* (1996)
AH204	JB436 R1162 (Str^r)	This study
Plasmids	**Description**	**Reference**
pBR322	Tet^r, Amp^r	Bolivar *et al.* (1977)
pAH10	Cm^r, Tet^s, Amp^s pBR322 derivative	This study
Jp103	*URA3, LEU2,* partial Tet^r	Laboratory plasmid
R1162	Str^r	Guerry *et al.* (1974)
pAH11	Cm^r, Str^s R1162 derivative	This study

With the exception of AH209, all strains created for this study were made by transforming competent cells of the relevant parental strain with purified plasmid DNA. Plasmid DNA was isolated using the PureLink® Quick Plasmid Miniprep Kit by Invitrogen (USA). Competent cells were prepared using calcium chloride and then transformed as described by Sambrook *et al.* (1989). AH209, a spontaneous nalidixic acid resistant mutant, was isolated by inoculating LB + Nx agar with approximately 10^8 cells of LU1. Potential mutants were then streaked to single colonies onto LB + Nx agar then LB agar and then LB + Nx agar again to confirm resistance.

The pBR322 plasmid contains the genes for ampicillin (*bla)* and tetracycline (tet^r) resistance and has a copy number of approximately 15-20 per cell (Covarrubias *et al.*, 1981). pAH14 was created by removing a 350 base pair section of pBR322 through digestion with *Hind*III and *Bam*HI. The plasmid was then blunted using T4 DNA polymerase and religated. The chloramphenicol acetyl transferase gene (*cat*) from pACYC184 was then inserted into the pBR322 *bla* gene at the *Pst*I restriction site to create pAH14.

Jp103 is a pUC replicon derived from the pKT2 plasmid and contains the *LEU2* gene from YEp13 (Broach *et al.*, 1979), the *URA3* gene from YEp24 (Botstein *et al.*, 1979), and the tet^r gene (J. A. Heinemann, personal communication). However, the beginning of the tet^r gene is interrupted by the insertion of the yeast *URA3* gene and as such the level of tetracycline resistance conferred is lower than that of pBR322 (J. A. Heinemann, personal communication).

R1162, also known as RSF1010, is a plasmid that contains genes conferring resistance to streptomycin and sulfonamide, has a copy number of approximately 10 per cell and is in the IncP-4 group (Barth & Grinter, 1974; Guerry *et al.*, 1974; Meyer *et al.*, 1982). pAH11 is a

derivative of R1162 with the *cat* gene from pACYC184 inserted between the *Eco*RI and *Not*I sites, replacing the streptomycin resistance genes.

LB agar supplemented with the appropriate antibiotics was used for all experiments except when selection of AH206 was required. For the competitions involving AH206, media impermissive to growth of the leu^- ura^- auxotrophic host without the plasmid was used to select for this strain. All dilution series were performed in phosphate buffered saline (PBS). The medium was synthetic complete selective agar without leucine or uracil (SC-L-U). This was made using 15 g/L agar (Oxoid, UK), 20 g/L glucose, 6.7 g/L Difco Yeast Nitrogen base (without amino acids), 160 mg/L proline, 20 mg/L arginine, 30 mg/L tyrosine, 200 mg/L threonine (from Applichem, Germany), 20 mg/L adenine, 30 mg/L lysine, 20 mg/L methionine, 50 mg/L phenylalanine, 20 mg/L histidine, and 20 mg/L tryptophan (from Sigma-Aldrich, USA).

4.2.2 Confirming the selective traits of each strain

Before any competition experiments were performed, each strain was tested based on genotype to ensure that they could be distinguished by growth on different media. Each strain was grown in a liquid culture in LB to $OD_{600} \approx 1$. Cultures were then used to inoculate LB agar, the appropriate selective agar, and the selective agar for the relevant competing strain. Plates were inoculated with the bacteria at varying dilutions 10 µl droplets (referred to as 'spot plates', as described in Section 2.2.2) or 100 µl of the culture was spread on a plate. Bacteria were centrifuged, resuspended in PBS and diluted in PBS before the SC-L-U agar plates were inoculated. This step was necessary to avoid contaminating the SC-L-U plates with any leucine or uracil that is present in LB. Plates were incubated overnight at

37°C before colonies were counted. This was used to calculate an EOP as described in Figure 2.1. Each test was performed three times.

4.2.3 Determining the response of each strain to herbicides and antibiotics

The MIC of the relevant herbicide was determined for each strain using the method described in Section 2.2.2. A herbicide concentration that had no effect on growth was then chosen, if possible at the same concentration as in the previous study by Kurenbach *et al.* (2015). A killing curve was derived from the growth of each strain in the presence of the herbicide and varying antibiotic concentrations compared to the antibiotic only, as described in section 2.2.2. Briefly, a series of LB agar plates containing the antibiotic at increasing concentrations with and without the herbicide were poured and left to dry for at least an hour in a laminar flow hood. Once dry, they were inoculated with 10µl drops of a saturated culture of *E. coli* at various dilutions representing 10^2 to 10^8 of the original culture. Once these spots had dried, plates were incubated at 37°C for up to four days with colonies counted each day. These killing curves were performed to ensure that each strain displayed the same decreased tolerance to the antibiotic in the presence of the herbicide as was previously observed. These experiments were also used to determine the appropriate antibiotic and herbicide concentrations to use in the competition experiments.

4.2.4 Competition experiments

Competition experiments were performed using isogeneic strains of *Escherichia coli* that differed in their level of resistance to a specific antibiotic with an additional unique selectable marker (Table 4.2). Strains were mixed together in equal proportions and

incubated with aeration in liquid LB medium until the culture increased by approximately 40 generations. Each competition was performed under eight different conditions: LB only, three increasing herbicide concentrations, antibiotic only, and each herbicide concentration with the antibiotic (Table 4.3). The proportion of each strain over time was measured to determine which conditions influenced the competition between strains. The antibiotic and herbicide concentrations used were intended to be low enough that there was no selection in favour of either strain when only exposed to one chemical.

Table 4.2 – Strains used in each competition experiment.

Medium	Resistant Strain	Susceptible Strain
Tetracycline and Roundup		
Competition 1	AH205 (Amp)	AH206 (SC-L-U)
Competition 2	AH206 (SC-L-U)	AH209 (Nx)
Competition 3	AH201 (Amp)	AH214 (Cm)
Streptomycin and Kamba		
Competition 4	AH204 (Nx)	AH211 (Cm)

Brackets represent the selectable marker for each strain. These supplements were added to the medium to select for specific strains after the competition. All strains were cultured on LB agar + the relevant antibiotic with the exception AH206 where a defined medium lacking leucine and uracil (SC-L-U) was used.

Table 4.3 – Antibiotic and herbicide concentrations used in each competition.

	Antibiotic Concentration (μg/mL)	Herbicide Concentrations (ppm ae)		
Competition 1	5	12	62	622
Competition 2	0.1	12	62	622
Competition 3	0.05	12	62	311
Competition 4	0.25	18	183	1371

Competitions 1-3 were performed using the antibiotic tetracycline and the herbicide Roundup. Competition 4 was performed using the antibiotic streptomycin and the herbicide Kamba.

Each strain was cultured to saturation in LB medium inoculated by a single colony on a streak plate. The OD_{600} of each culture was measured and used to calculate an approximate density. Based on the OD_{600} reading 1 x 10^9 cells were pelleted by centrifugation and

resuspended in 1mL of fresh LB. This step was performed so that each culture had an equal concentration of bacteria and to remove the antibiotics in the culture medium. Each strain was diluted 1000-fold and 50 µl of this dilution added to every condition in which the competition was performed. At this point a titre of each strain was also determined so the starting proportions could be accurately calculated.

Competitions were carried out in 10mL of LB medium supplemented with the appropriate concentrations of herbicide, antibiotic, both or neither (Table 4.3). Once both strains had been added in approximately equal amounts, each treatment incubated at 37°C with rotation providing aeration for 24 hours. After 24 hours, a 1000-fold dilution was made into 10 mL of fresh LB medium under the same conditions. These cultures were then incubated at 37°C for another 24 hours before the sub-culturing was repeated. This cycle continued four times.

At the end of every growth period, each competition culture was plated in triplicate on LB and the selective medium for each strain in the competition to enumerate the competitors. Plates were inoculated with 10 µl drops of each competition culture at various dilutions ranging from 10^2 to 10^8. Additional plates were inoculated with 100 µl of each culture, giving a 10-fold dilution, lowering the detection limit. When the competition involved strain AH206, 1mL of culture from each condition was pelleted by centrifugation and resuspended in PBS. PBS was then used for the dilution series as the leucine and uracil present in the LB medium was sufficient for both strains to grow on SC-L-U agar rather than just AH206. For all other competitions LB medium was used for the dilution series. Plates were incubated for approximately 16 hours at 37°C before colonies were counted. As colonies grew slower on the SC-L-U plates, these were incubated for another 24 hours before counting.

The growth on the selective media was used to determine the proportion of each strain present at the end of each day of the competition. The proportions were determined by dividing the count of one strain by the total count of the two strains in the competition. The proportions were tracked over the course of the experiment and this data was used to calculate the strength of selection. The strength of selection was assumed to be the difference in the exponential growth rate of the two strains (Bosch *et al.*, 2014; Otto & Day, 2007). Under this interpretation, the change in the frequency of resistant individuals per unit of time is the logistic curve, the explicit solution is shown in Figure 4.1 (Mallet, 2012). This formula was rearranged to give the formula for calculating the strength of selection in Figure 4.3.

$$p = \frac{e^{st}}{c + e^{st}}$$

Figure 4.1 – Equation for the change in frequency of resistant individuals per unit time.
Where t = time, p = frequency of resistant individuals, s = strength of selection, c = constant describing the initial frequency of resistant individuals (Figure 4.2).

$$c = \frac{1}{p_0} - 1$$

Figure 4.2 – Equation for the constant describing the initial frequency of resistant individuals.
Where p_0 is the frequency of resistant individuals at t_0.

$$s = \frac{\ln\left(\frac{pc}{1-p}\right)}{t}$$

Figure 4.3 – Equation for calculating the strength of selection in favour of the resistant strain.
Where p = the proportion of resistant individuals at time t, c = the constant describing the initial frequency of resistant individuals (Figure 4.2), t = time, and s = strength of selection.

4.2.5 Growth Curves

The strains used in competitions 3 & 4 were cultured in isolation in four of the competition conditions: LB only, herbicide, antibiotic, and antibiotic + herbicide. Only the highest herbicide concentration was tested, as this had the greatest effect on the strength of selection. 24-well plates containing 1 mL of LB medium were inoculated with 10 µl of a saturated culture, and the relevant concentrations of herbicide and/or antibiotic. Cultures were grown for 16 hours at 37°C with rotation providing aeration. A FLUOstar® Omega microplate reader (BMG LABTECH, Germany) was used to measure the OD_{600} of each well every six minutes with 22 flashes per well, averaged using orbital averaging set to 8mm. The OD_{600} measurements from each time point were averaged across the five replicates and the change in OD_{600} over time used to create a growth curve for each strain in each condition.

4.2.6 Statistical analysis

R was used for all statistical analysis (R Core Team, 2015). For the killing curve assay the statistical analysis described in section 2.2.5 was used. For the competition experiments, a univariate ANOVA was performed to calculate contrasts between levels of interest. Each treatment combination was a different level and so it was possible to determine whether herbicides and/or antibiotics changed the strength of natural selection on resistant genotypes. Contrasts were used to determine if the presence of antibiotics significantly changed the strength of selection when herbicide concentrations were constant, and whether treatments containing herbicide only or antibiotic only had a significantly different strength of selection when compared to the LB control. These were tested using the glht function in the multcomp package (Hothorn *et al.*, 2008) with a two-sided alternative and

sequential Bonferonni procedure (Holm, 1979). For these analyses, residual plots were used to test for normality and equality of variance.

4.3 Results

4.3.1 Selective markers of each strain

All strains were tested to ensure that they could grow on the selective medium to the same density as on non-selective medium and that they were unable to grow on the selective media designed for the opposing strain in the competitions. The EOP of each strain on media intended to select for it and the relevant opposing strain was calculated (Table 4.4). The EOP is a measure of relative survival with the cfu/mL able to survive on a given medium compared to the cfu/mL that survive on LB (Figure 2.1). An EOP of 1 indicates the strain grows to the same density as on LB.

Table 4.4 – EOP of different *E. coli* strains on selective media.

		EOP on media selecting the:	
Medium	**Strain**	**Resistant Strain**	**Susceptible Strain**
LB + Amp	AH205 (R)	1.1 ± 0.1	$2.6 \times 10^{-8} \pm 1.7 \times 10^{-8}$
SC-L-U	AH206 (S)	$1.5 \times 10^{-8} \pm 0.4 \times 10^{-8}$	1.1 ± 0.4
SC-L-U	AH206 (R)	1.1 ± 0.4	$2.7 \times 10^{-8} \pm 0.4 \times 10^{-8}$
LB + Nx	AH209 (S)	$2.3 \times 10^{-8} \pm 0.4 \times 10^{-8}$	1.2 ± 0.2
LB + Amp	AH201 (R)	1.1 ± 0.1	$1.5 \times 10^{-8} \pm 0.3 \times 10^{-8}$
LB + Cm	AH214 (S)	$1.2 \times 10^{-8} \pm 0$	1.1 ± 0.1
LB + Nx	AH204 (R)	1.0 ± 0.2	$1.1 \times 10^{-8} \pm 1.5 \times 10^{-8}$
LB + Cm	AH211 (S)	$1.7 \times 10^{-7} \pm 0.6 \times 10^{-7}$	1.1 ± 0.4

Media supplements to select for each strain are outlined in Table 4.2. R indicates the strain is more resistant; S indicates the strain is more susceptible in the competition. EOP values are the average of three independent experiments ± SEM.

The appropriate selective medium can accurately distinguish between the resistant and susceptible strains used in each competition (Table 4.4). In all cases, the growth of a strain on its appropriate selective medium had no effect on the cfu/mL compared to LB, as shown by the EOP ≈ 1. In the opposite scenario, where a strain was grown on the selective medium intended to select for the competition partner, very little growth was observed, as indicated by EOP values around or below the detection limit. In these cases, the variation in EOP values is the result of different LB titres between replicates.

4.3.2 The effect of herbicides and antibiotics on each strain

The MIC of the relevant herbicide for each of the strains used in the competition experiments was determined (Table 4.5). This was used to identify a concentration that would not have any impact on growth in experiments containing both the herbicide and antibiotic. It was also necessary to determine a concentration that would not change the fitness of either strain in a competition experiment.

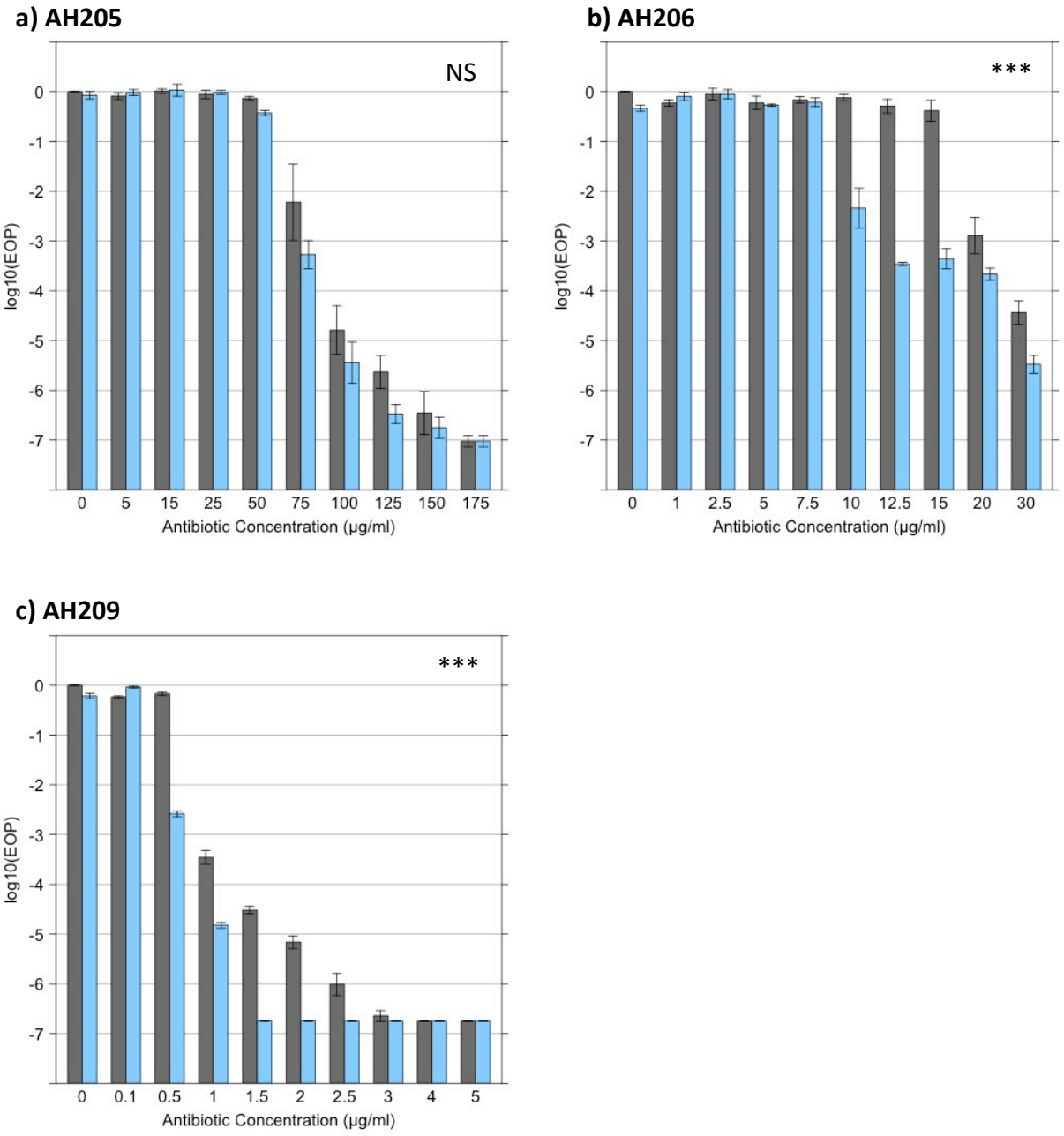
Table 4.5 – Minimum inhibitory concentrations of herbicide formulations.

Strain	Roundup MIC (ppm ae)	Kamba MIC (ppm ae)
AH205	1,990 ± 0	-
AH206	1,990 ± 0	-
AH209	1,740 ± 0	-
AH201	1,250 ± 0	-
AH214	1,500 ± 0	-
AH204	-	11,580 ± 610
AH211	-	9,750 ± 610

MICs ± SEM (n=3), values are rounded to the nearest 10 ppm ae.

Each strain was also individually tested to determine whether exposure to the herbicide and antibiotic resulted in a change in EOP that differed from the antibiotic only treatment, as seen by Kurenbach *et al.* (2015) (Figure 4.4 & Figure 4.5).

Figure 4.4 – Killing curves for the strains used in Competitions 1 & 2.

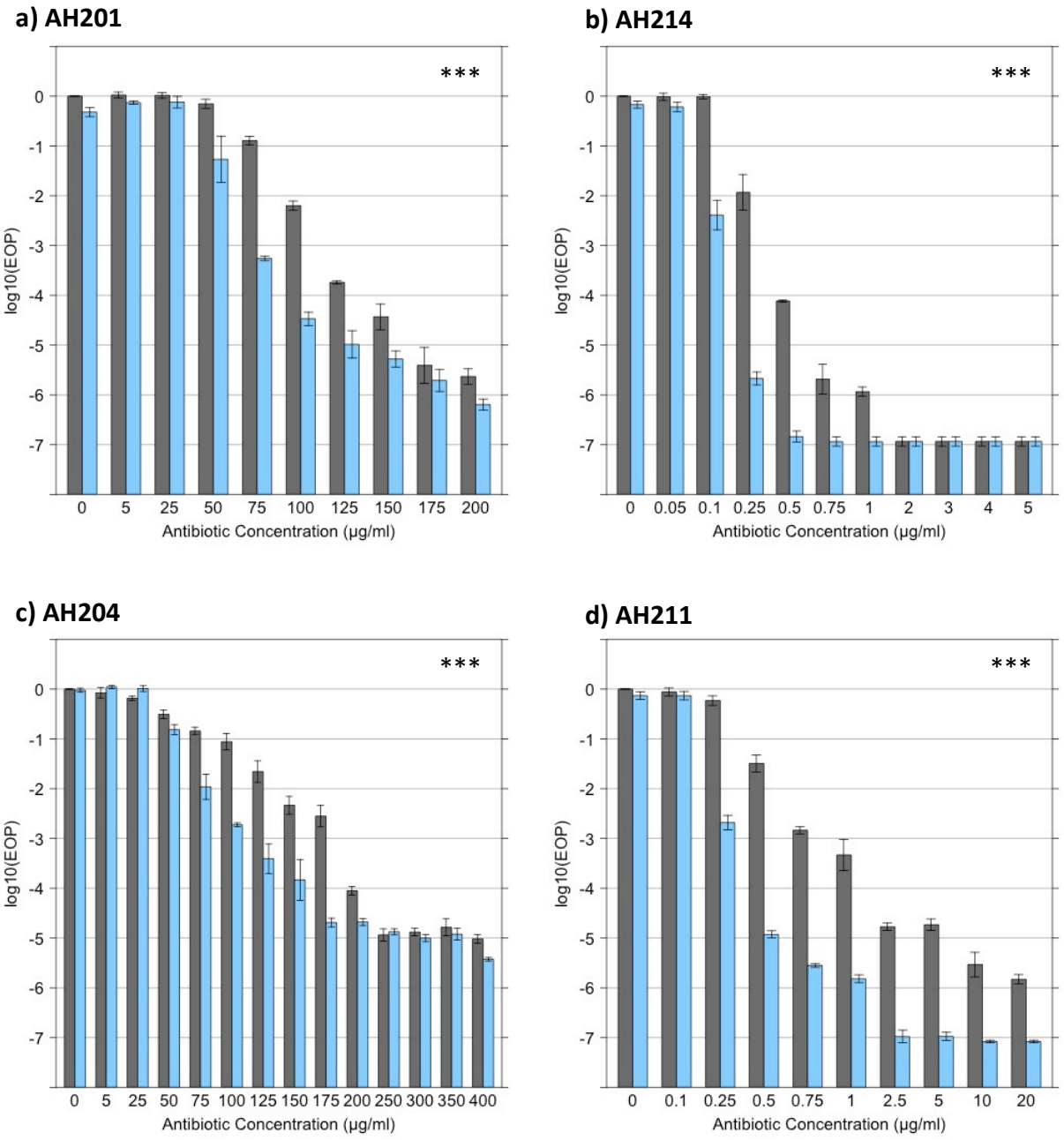


Survival of different *E. coli* strains on a range of concentrations of tetracycline with (blue) and without (grey) 622 ppm ae Roundup. Survival is reported as log-transformed EOP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

The EOP of AH205 was not significantly ($p = 0.3995$) changed by Roundup (Figure 4.4a). This strain was resistant to the highest concentrations of tetracycline (MIC = 100 μg/mL). The EOP of AH206 was significantly ($p = 1.04 \times 10^{-14}$) different when it was exposed to Roundup (Figure 4.4b). This strain was resistant to tetracycline concentrations intermediate between AH205 and AH209 (MIC = 30 μg/mL). There was a significant decrease in the EOP at tetracycline concentrations between 12.5 and 15 μg/mL when Roundup was present. This resulted in the MIC being 2.4-fold lower when Roundup was present compared to when it was absent.

The EOP of AH209, which had the lowest tetracycline MIC (1.5 μg/mL), was significantly ($p < 2 \times 10^{-16}$) further decreased when Roundup was present. This corresponds to a 1.5-fold decrease in MIC. A three-fold decrease in tolerance observed for this combination by Kurenbach *et al.* (2015). However, the experiment in this thesis was performed using a different strain and different antibiotic concentrations making comparisons difficult (Rosner, 1985). In general, exposure to the herbicide Roundup still induced a decrease tetracycline tolerance, even in *E. coli* with acquired resistance. These killing curves were also used to determine antibiotic concentrations that would not alter the fitness of competitors in the competition experiments.

Figure 4.5 – Killing curves for the strains used in Competitions 3 & 4.



Survival of different *E. coli* strains on a range of antibiotic concentrations with (blue) and without (grey) herbicide. AH201 and AH214 killing curves were performed using tetracycline and 622 ppm ae Roundup, AH204 and AH211 killing curves used streptomycin and 1,371 ppm ae Kamba. Survival is reported as log-transformed EOP scores, error bars are SEM (n=3). Asterisks correspond to significance level: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

All of the strains used in Competitions 3 & 4 exhibited a significant decrease in antibiotic tolerance when exposed to the relevant herbicide and antibiotic (Figure 4.5). The EOP of AH201 was significantly ($p = 6.61 \times 10^{-8}$) decreased by Roundup. This strain was resistant to

high tetracycline concentration (MIC = 125 µg/mL); Roundup reduced the MIC by 1.7-fold. The EOP of AH214 was significantly decreased ($p < 2 \times 10^{-16}$) when Roundup was present. The MIC of tetracycline dropped from 0.5 µg/mL to 0.25 µg/mL when Roundup was present, a 2-fold decrease in MIC. However, the tetracycline concentrations involved are far smaller as this strain has no acquired tetracycline resistance. AH201 and AH205 have different reactions upon addition of tetracycline and Roundup but harbour the same plasmid. This indicates the mechanism is not plasmid encoded but related to the strain.

The EOP of AH204 was significantly reduced ($p = 1.57 \times 10^{-13}$) when Kamba was present. This strain was resistant to high streptomycin concentrations (MIC = 200 µg/mL), however a 1.6-fold decrease in MIC occurred when Kamba was present. The EOP of AH211 also was also significantly ($p < 2 \times 10^{-16}$) reduced when Kamba was present. This strain has a comparatively low streptomycin MIC (1 µg/mL), a 2-fold reduction in MIC occurred when Kamba was present. The concentrations used in the AH211 killing curve assay are approximately 100 times lower than those for AH204 confirming the differing resistance levels. Antibiotic concentrations that caused no change in EOP were derived from the killing curves and used in the competition experiments.

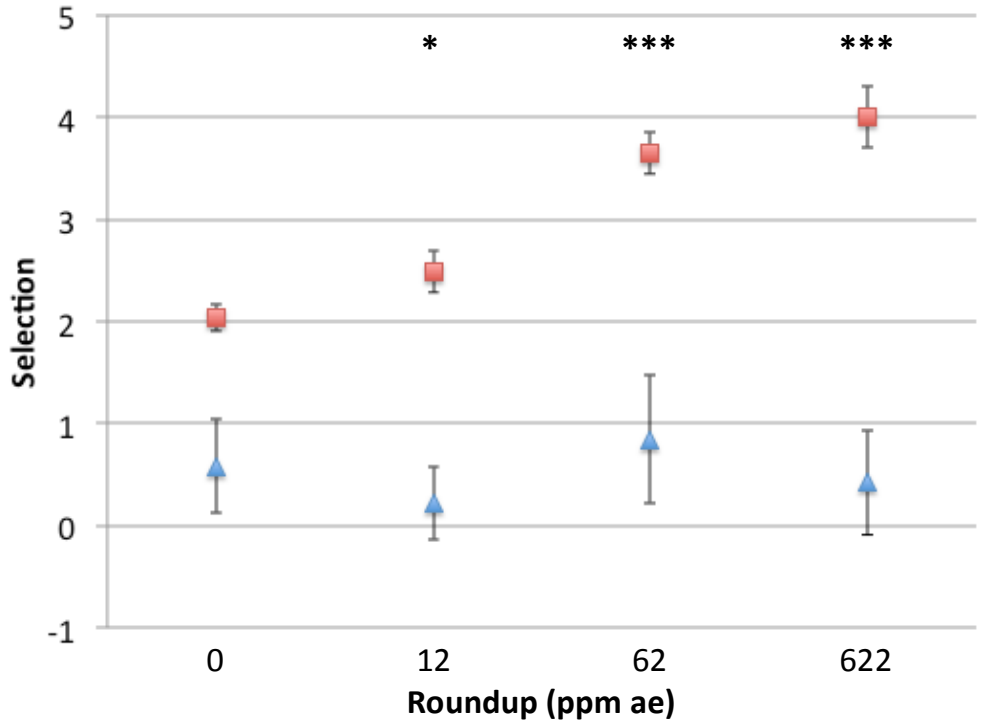
4.3.3 Competition experiments

The proportion of each strain was measured at regular intervals following competition in eight conditions. The strength of selection in favour of the resistant strain was then calculated based on the change in proportion. Antibiotic and herbicide concentrations used were intended to be low enough that the presence of only one chemical would not change the fitness of either strain. The key test of these experiments was how the proportions of

each strain would shift when exposed to the herbicide and antibiotic together, in particular whether this would be sufficient to cause selection in favour of the resistant strain.

Competition 1 used the resistant strain AH205 and the susceptible strain AH206. The strength of selection in favour of AH205 was derived from the change in proportion of AH205 (Figure 4.6). An increase in the proportion of the resistant strain over time will lead to a positive selection value, while an increase in the proportion of the susceptible strain will lead to a negative selection value.

Figure 4.6 – Competition 1: Strength of selection in favour of AH205 following competition with AH206.



Strength of selection was calculated using the equation in Figure 4.3. Squares indicate treatments that contained 5 µg/mL tetracycline, triangles indicate treatments containing no tetracycline. Results are the average of three independent experiments. Error bars are SEM. Asterisks indicate contrasts where the presence of the antibiotic significantly changed the strength of selection: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

There was a significant increase in the strength of selection in each of the tetracycline and Roundup treatments when compared to the relevant Roundup only treatments for Competition 1. While differences were observed in the strength of selection in favour of the

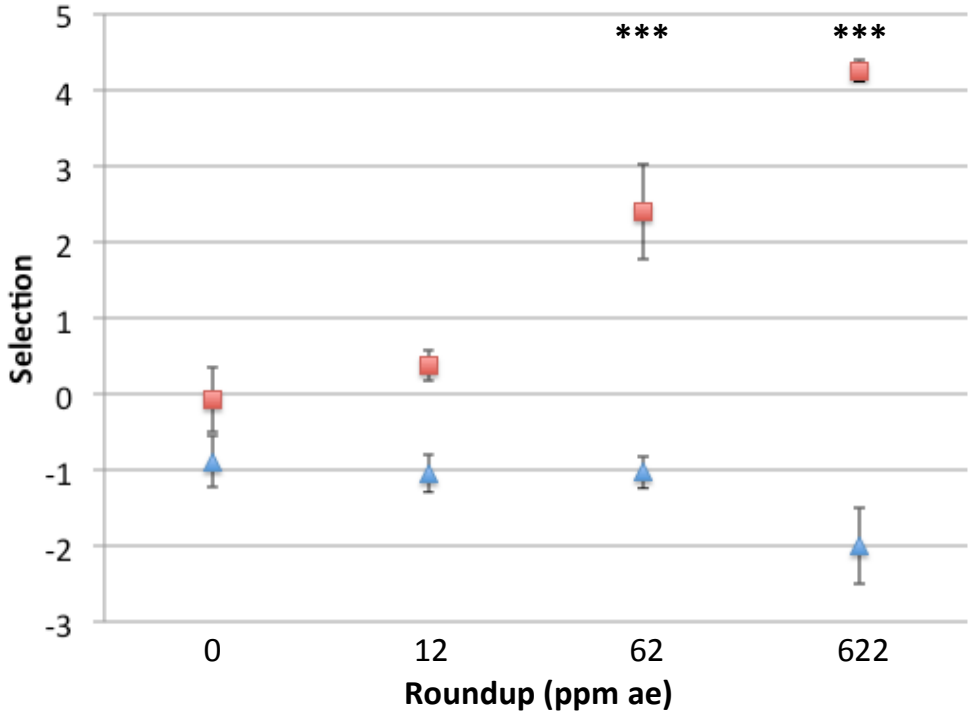
resistant strain between the LB (Figure 4.6, leftmost blue triangle) and tetracycline only (Figure 4.6, leftmost red square) conditions, this was not significant ($p = 0.0833$). There were no significant differences in selection strength observed when each herbicide concentration (Figure 4.6, three rightmost blue triangles) was compared to the LB control (Figure 4.6 leftmost blue triangle). The susceptible strain decreased in proportion in all conditions after four days, which indicates that the susceptible strain was less fit than the resistant strain. If the strains were equally fit the proportion of each strain was expected to remain at approximately 50% in the LB and Roundup only treatments. The large error bars indicate that there was a high degree of variability in the strength of selection between replicates.

When each of the herbicide and antibiotic conditions was compared to the antibiotic only condition (Figure 4.6, leftmost red square), only the Roundup 622 ppm ae + tetracycline condition (Figure 4.6, rightmost red square) was significantly different to the tetracycline only treatment ($p = 0.019$). The presence of Roundup significantly increases the strength of selection in favour of the more resistant strain within the competition at an antibiotic concentration that in isolation has no significant effect. As the herbicide concentration decreases so to does the strength of selection in favour of the resistant strain.

In Competition 2 between AH206 and AH209 the tetracycline concentration was decreased to compensate for the low tetracycline MIC of AH209. There was a significant increase in selection in favour of AH206 in both tetracycline + Roundup 62 ppm ae ($p = 5.97 \times 10^{-5}$) and tetracycline + Roundup 622 ppm ae ($p = 2.49 \times 10^{-8}$) when compared to the relevant Roundup only treatments (Figure 4.7). These two antibiotic + herbicide treatments were also significantly different from the antibiotic only treatments. There were no significant

differences between the antibiotic only and herbicide only treatments when compared to the LB control.

Figure 4.7 – Competition 2: Strength of selection in favour of AH206 following competition with AH209.



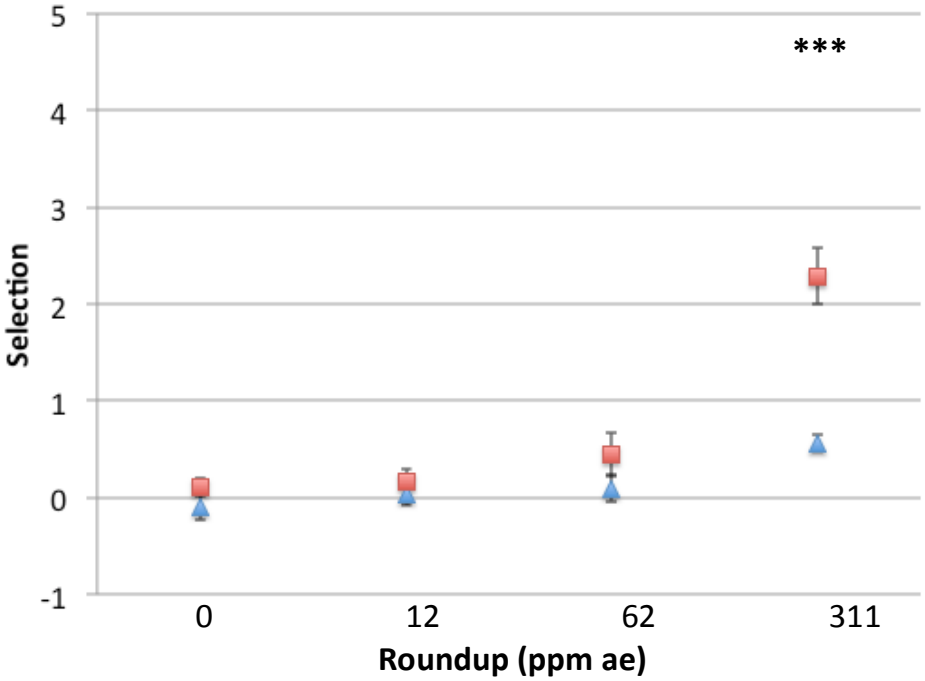
Strength of selection was calculated using the equation in Figure 4.3. Squares indicate treatments that contained 0.1 µg/mL tetracycline, triangles indicate treatments containing no tetracycline. Results are the average of three independent experiments. Error bars are SEM. Asterisks indicate contrasts where the presence of the antibiotic significantly changed the strength of selection: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

AH206 appeared to be less fit than AH209. This is indicated by the negative strength of selection values for the LB and herbicide only treatments (Figure 4.7, blue triangles), caused by the susceptible strain having increased in proportion by the end of the competition. In fact, it took 0.1 µg/mL of tetracycline for the resistant strain to overcome the fitness difference and reach an equilibrium where there was little change in the proportion of either strain by the end of the competition (Figure 4.7, leftmost red square). This difference in fitness highlights how large an impact the addition of tetracycline can have when comparing the strength of selection between Roundup only and Roundup + tetracycline treatments. This fitness difference may be caused by the different plasmid carried by

AH206 because it is both larger, and replicates to a higher copy number plasmid than the plasmid in AH205. AH209 did not harbour any plasmids.

Given the differences in fitness between the strains, I designed a new series of strains to make a better test of the hypothesis that in a competition between isogenic strains with differing levels of tetracycline resistance, tetracycline and Roundup could cause an increase in the strength of selection in favour of the resistant strain at an antibiotic concentration that in isolation had no effect on selection. Competition 3 tested this hypothesis using AH201, the resistant strain, and AH214, the susceptible strain (Figure 4.8).

Figure 4.8 – Competition 3: Strength of selection in favour of AH201 following competition with AH214.



Strength of selection was calculated using the equation in Figure 4.3. Squares indicate treatments that contained 0.05 µg/mL tetracycline, triangles indicate treatments containing no tetracycline. Results are the average of three independent experiments. Error bars are SEM. Asterisks indicate contrasts where the presence of the antibiotic significantly changed the strength of selection: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

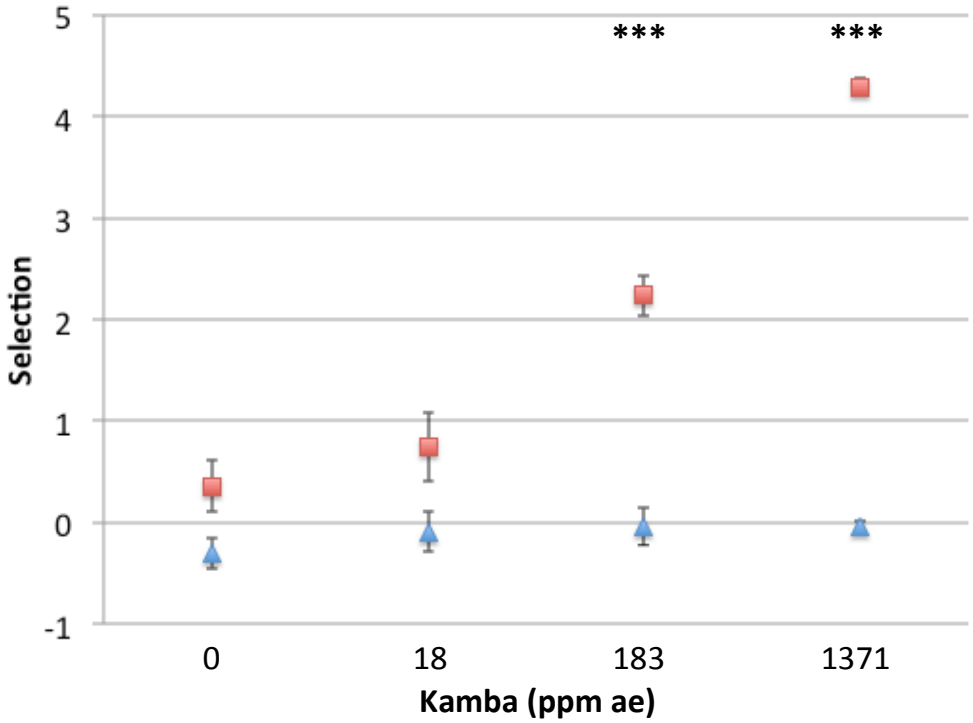
In this case, only the highest Roundup concentration, 311 ppm ae, in combination with tetracycline resulted in a significant increase in selection strength when compared to both the Roundup only treatment ($p = 1.70 \times 10^{-5}$) and the tetracycline only treatment ($p = 8.28 \times$

10^{-7}). The highest Roundup concentration (Figure 4.8, rightmost blue triangle) alone caused slight selection in favour of the resistant strain, AH201, however this was not significant when compared to the LB control (Figure 4.8, leftmost blue triangle) ($p = 0.11$). The Roundup 12 ppm ae, Roundup 62 ppm ae and tetracycline conditions caused no significant changes in selection when compared to the LB control. As expected, the strains were equally fit in LB. Also, the lower Roundup + tetracycline treatments caused no significant change in selection when compared to the antibiotic or herbicide only treatments, indicating that this response is dependent on the herbicide concentration used. As this is reduced, so is the selective pressure in favour of the resistant strain in the absence of any other environmental changes.

The conditions for competition 4 used the antibiotic streptomycin and the herbicide Kamba (Figure 4.9). There were no significant changes in the strength of selection observed in mixed cultures in either sub-lethal concentrations of streptomycin or Kamba, when compared to culture in LB. When both streptomycin and Kamba were part of their environment, some significant changes in the strength of selection in favour of AH204, the streptomycin resistant strain, were observed.

The combination of herbicide and antibiotic was significantly different to herbicide alone. Kamba 183 ppm ae and streptomycin together created a selection strength significantly greater for AH204 than either Kamba 183 ppm ae only ($p = 5.29 \times 10^{-6}$) or streptomycin only ($p = 4.92 \times 10^{-5}$). The same effect was observed at the highest Kamba concentration (1371 ppm ae) with streptomycin where selection in favour of the resistant strain was significantly higher than the Kamba 1371 ppm ae control ($p = 7.00 \times 10^{-10}$) and the streptomycin only control ($p = 2.47 \times 10^{-9}$).

Figure 4.9 – Competition 4: Strength of selection in favour of AH204 following competition with AH211.



Strength of selection was calculated using the equation in Figure 4.3. Squares indicate treatments that contained 0.25 µg/mL streptomycin, triangles indicate treatments containing no streptomycin. Results are the average of three independent experiments. Error bars are SEM. Asterisks indicate contrasts where the presence of the antibiotic significantly changed the strength of selection: (NS) not significant, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

The decrease in tolerance caused by herbicides is sufficient to increase the fitness of resistant bacteria at an antibiotic concentration that otherwise would be neutral to competition with susceptible strains.

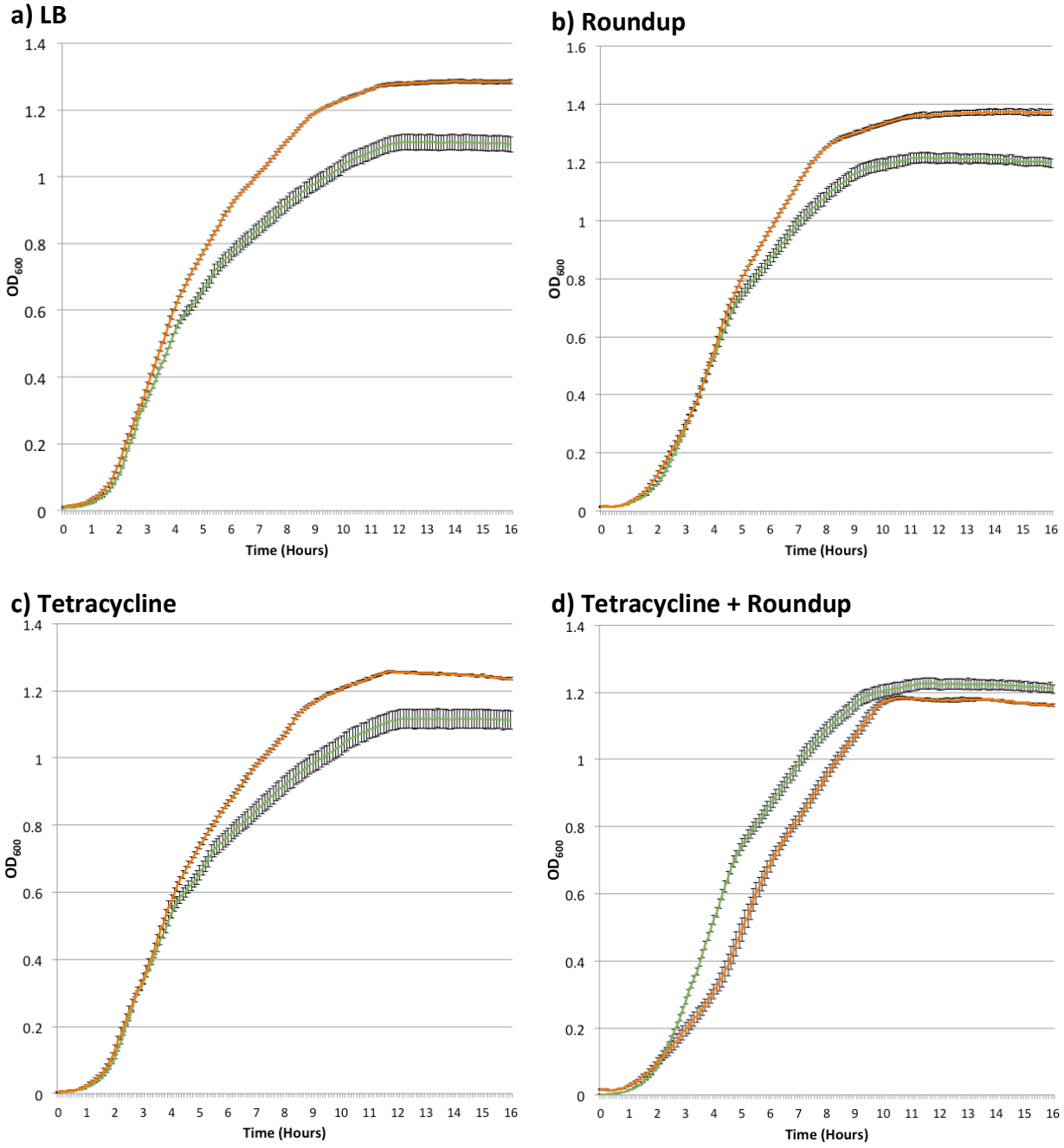
4.3.4 Growth curves

The strains used in competitions 3 & 4 were grown in isolation to determine whether the changes in strength of selection were due to an increased generation time of the susceptible strains or if no growth occurred at all. Each strain was cultured in LB, the antibiotic, the highest herbicide concentration tested and the herbicide + antibiotic. Growth curves were derived from the OD_{600} data over 16 hours.

In the LB, tetracycline, and Roundup only conditions of competition 3 the susceptible strain, AH214, was able to grow to a higher final optical density after 16 hours (Figure 4.10). These cultures grew at a very similar rate during their exponential growth phase in each of these treatments. The difference between the two strains was only evident in the tetracycline + Roundup condition where AH201 was able to grow to a higher optical density more quickly than AH214. This shows that the difference in proportion of the two strains measured at the end of the competition was likely due to a shorter generation time for AH201.

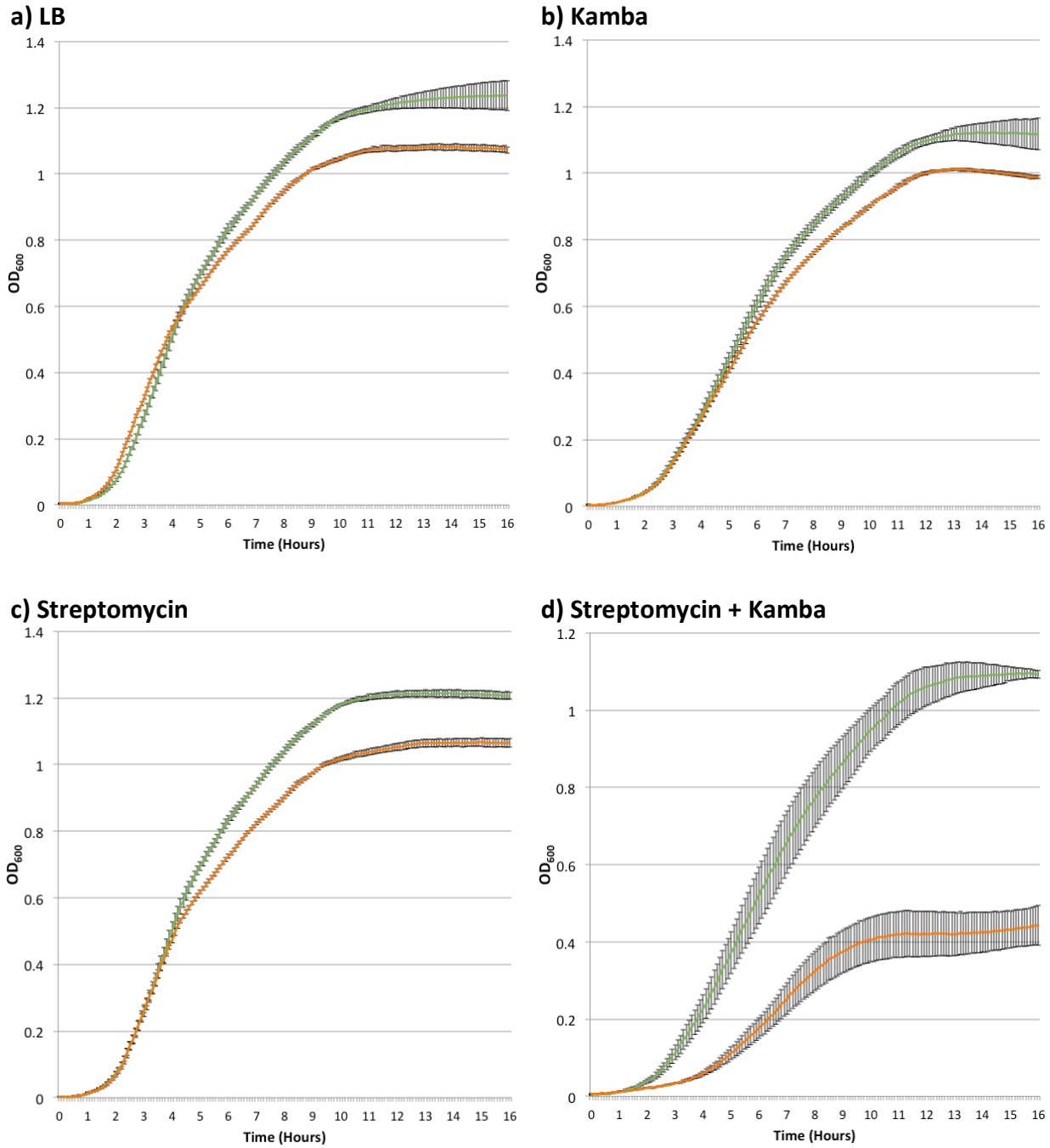
In competition 4, the more resistant strain, AH204, reached higher population densities but had a very similar exponential growth rate to AH211 in the LB, Kamba only and streptomycin only conditions (Figure 4.11). This was as expected from the competition experiments where there was no change in the strength of selection in favour of the resistant strain in any of these treatments. The main difference between the two strains occurred in the streptomycin and Kamba condition where the resistant strain, AH204 reached a similar population density at a similar time as in the other treatments while AH211 reached a much lower final density. This indicates that the susceptible strain can reproduce to some extent in the treatment but it is far slower than the resistant strain.

Figure 4.10 – Growth curves for AH201 and AH214 in four treatments.



Growth of AH201 (green) and AH214 (orange) in four different treatment conditions. Each curve shows the average of five independent experiments, error bars are SEM. When present, the tetracycline concentration was 0.05 µg/mL and the Roundup concentration was 311 ppm ae.

Figure 4.11 – Growth curves for AH204 and AH211 in four treatments.



Growth of AH204 (green) and AH211 (orange) in four different treatment conditions. Each curve shows the average of five independent experiments, error bars are SEM. When present the streptomycin concentration was 0.25 µg/mL and the Kamba concentration was 1371 ppm ae.

4.4 Discussion

How bacteria resist antibiotics and how the environment selects resistant bacteria have been extensively studied, but there is still more to learn (The Review on Antimicrobial Resistance, 2016). There are many environmental stressors that can cause adaptive changes in antibiotic tolerance, including: pH and anaerobiosis (Fernández *et al.*, 2011), non-antibiotic pharmaceuticals such as chlorpromazine (Kristiansen *et al.*, 2010), biocides such as trisodium phosphate, sodium nitrite and sodium hypochlorite (Molina-Gonzalez *et al.*, 2014), quaternary ammonium compounds (Webber *et al.*, 2015), and triclosan (Braoudaki & Hilton, 2004). This thesis and previous works have shown that commercial herbicide formulations and their active ingredients are able to induce increases in antibiotic tolerance that can lead to a higher frequency of acquired resistance (Kurenbach *et al.*, manuscript in preparation; Kurenbach *et al.*, 2015). Unique to this thesis is the demonstration that both increases and decreases in antibiotic tolerance induced by herbicides can accelerate the evolution of acquired resistance.

4.4.1 Commercial herbicide formulations can decrease the antibiotic tolerance of E. coli

Two herbicide and antibiotic combinations were focused on for these experiments: tetracycline + Roundup and streptomycin + Kamba. Similar combinations have already been shown to cause a decreased tolerance of *E. coli* to some antibiotics (Gibson, 2016; Kurenbach *et al.*, 2015). The results presented here were consistent with those previously reported by Kurenbach *et al.* (2015) and Gibson (2016). Different strains of *E. coli* and different herbicide concentrations were used between these experiments making direct

comparisons difficult, however, a significant decrease in antibiotic tolerance upon herbicide exposure was observed for all antibiotic susceptible strains. The MIC of tetracycline decreased by 1.5-fold and 1.6-fold when Roundup was present in AH209 and AH214 respectively, slightly lower than the 3-fold decrease observed by Kurenbach *et al.* (2015). The MIC of streptomycin decreased by 2-fold when Kamba was present in AH211, similar to what was observed by Gibson (2016).

Interestingly, in three out of the four antibiotic resistant strains, a significant decrease in antibiotic tolerance was still observed. This indicates that the mechanism through which these adaptive changes occur was separate to any mechanism harboured by the plasmids. Roundup had no significant effect on the MIC of AH205, which harboured the pBR322 plasmid with the *tet*^{r} gene. However, Roundup did cause a 1.7-fold decrease in the MIC of tetracycline in AH201. This is interesting as AH201 and AH214 harbour the same plasmid; the only difference is the strain background with AH205 originating from LU1 (RR1 ancestry) and AH201 from SB21 (C600 ancestry). Further study of the differences between these two strains could give an insight into the mechanism through which Roundup causes this decrease in tetracycline tolerance. A comparison of the genome of each strain could identify genes that are present in only one strain along with potential functions. After candidates that may be responsible for this effect had been identified, gene deletion mutants could be utilised to see the extent to which a given gene contributes to the decreased antibiotic tolerance caused by Roundup. Kamba lowered the MIC of streptomycin by 1.6-fold in AH204, the streptomycin resistant strain.

The mechanism through which the herbicides cause a decrease in antibiotic tolerance occurred is not fully understood. *E. coli* mutants with a specific gene deletion (Δ*acrA,* Δ*acrB,*

Δ*acrD,* Δ*tolC,* or Δ*ompF*) were used to investigate the role of efflux and influx in herbicide-induced adaptive changes in antibiotic tolerance (Kurenbach *et al.*, manuscript in preparation). OmpF is a non-specific outer membrane porin while AcrAB-TolC and AcrAD-TolC are two RND-efflux pumps in *E. coli* (Li & Nikaido, 2004). Kamba had a larger effect on the MIC of streptomycin in the Δ*acrA* strain, which lacks AcrA, a periplasmic membrane fusion protein, than the wild-type strain (Kurenbach *et al.*, manuscript in preparation). However, Roundup had no significant effect on the MIC of tetracycline in the Δ*acrA* strain suggesting that AcrA is involved in the mechanism by which Roundup alters the susceptibility of *E. coli* to tetracycline (Kurenbach *et al.*, manuscript in preparation). For all other gene deletion mutants Roundup and Kamba still decreased the tolerance of *E. coli* to tetracycline and streptomycin respectively (Kurenbach *et al.*, manuscript in preparation). The magnitude of the decrease in tolerance was at times reduced indicating that targeted genes are likely involved in the response, however they are not the only explanation (Kurenbach *et al.*, manuscript in preparation).

A similar decrease in tolerance was observed when *E. coli* was exposed to salicylate and kanamycin (Aumercier *et al.*, 1990). Kanamycin is an aminoglycoside antibiotic as is streptomycin while salicylate is structurally similar to dicamba (Figure 3.1), the active ingredient in the Kamba formulation. It was hypothesised that the charge of the aminoglycoside antibiotics may be responsible for the decrease in antibiotic tolerance that was observed (Aumercier *et al.*, 1990). Antibiotics with a negative or neutral charge often enter a bacterial cell through the outer membrane porin OmpF, the expression of which is down regulated by salicylate (Cohen *et al.*, 1993; Foulds *et al.*, 1989). Aminoglycosides, however, are positively charged and are thought to diffuse into the cell directly through the

lipid bilayer (Nikaido, 2003). It was hypothesised that salicylate could increase the membrane potential allowing greater entry of the antibiotic into the cell (Aumercier *et al.*, 1990). Due to the structural similarities between Kamba and salicylate it is conceivable that the same mechanism is responsible for the decrease in streptomycin tolerance observed in this study.

4.4.2 Herbicides and antibiotics cause selection in favour of resistance

The seven strains created for this study were used to perform four competition experiments in the presence of either tetracycline + Roundup or streptomycin + Kamba. The purpose of these competition experiments was to determine if the addition of the herbicide to an environment that included low concentrations of antibiotic could change the relative fitness of resistant strains within a heterogeneous population. Competitions were started with an equal proportion of resistant and susceptible bacteria and the proportion at the end of the experiment was used to calculate the strength of selection acting on one of the strains. These values were then compared to each other in order to determine which treatments differed significantly.

The first two competitions were performed between a highly tetracycline resistant strain and a moderately tetracycline resistant strain and the same moderately resistant strain against a susceptible strain. In both competitions, the antibiotic and herbicide concentrations in isolation had no significant effect on the strength of selection in favour of the resistant strain. For competition 1, all three tetracycline + Roundup treatments were significantly different from their respective Roundup only treatment while only the highest tetracycline + Roundup treatment was significantly different from the tetracycline only treatment. For competition 2, the two highest tetracycline + Roundup treatments (62 and

622 ppm ae) were significantly different from both the antibiotic only treatment and their respective Roundup only treatment. The moderately tetracycline resistant strain used in both of these competitions was less fit than the competing strains in the absence of any selection and so these competitions were repeated with new strains.

In competition 3 there were no obvious differences in fitness in LB medium, herbicide only or antibiotic only as shown by the growth curves. Only at the highest Roundup concentration, 311 ppm ae, in combination with tetracycline was fitness of the more resistant strain significantly different compared to competition in the tetracycline only or Roundup only treatments. In competition 4, the two highest Kamba concentrations (183 ppm ae and 1371 ppm ae) in combination with streptomycin were the only conditions where selection in favour of the resistant strain was significantly increased compared to the Kamba only and streptomycin only controls.

The herbicide concentrations required to induce these effects were above the maximum residue limits for the Kamba active ingredient dicamba but are within the animal feed range for glyphosate, the active ingredient in Roundup (Codex Alimentarius Commision, 2016). All values were below the recommended application rates for the herbicide formulations (Tables 2.4 and 2.5). These results show that at herbicide concentrations commonly used in agriculture selection in favour of resistant bacteria can occur when antibiotics are also present.

Such combination exposures are expected to occur frequently. Antibiotics are commonly used in animal farming and large proportions of these are not metabolised by the animal and are subsequently excreted (Dolliver & Gupta, 2008; Kwon, 2011). In the USA, pig effluent is commonly disposed of through land application and tetracycline concentrations

of up to 46 mg/kg have been detected in pig manure (Hölzel *et al.*, 2010). Tetracycline is also used to prophylactically treat bee colonies (Tian *et al.*, 2012). Bees are commonly exposed to a wide range of pesticides with one study identifying 19 different pesticides in bees from Colorado (Hladik *et al.*, 2016). This included three herbicides, however the herbicides used in this thesis were not tested for (Hladik *et al.*, 2016).

The antibiotic concentrations used in these competitions were below the MIC of the relevant antibiotic for each strain. Previously it was thought that selection in favour of resistant bacteria only occurred in the concentrations between the MIC of the susceptible strain and the resistant strain, the "mutant selective window" (Drlica, 2003; Zhao & Drlica, 2001). This theory suggested that selection for resistant bacteria did not occur at concentrations below the MIC of the susceptible strain (Drlica, 2003; Drlica & Zhao, 2007). While it is true that antibiotic concentrations above MIC can select for resistant bacteria, as shown in Chapter 3, it does not tell the whole story. Recent work has shown that antibiotic concentrations far below MIC are still able to cause selection in favour of resistant bacteria (Gullberg *et al.*, 2014; Gullberg *et al.*, 2011). This work has determined that the minimum selective concentration (MSC) is often far below the MIC and that as soon as antibiotics begin to have an effect on the growth of susceptible bacteria, the resistant bacteria can begin to take over (Andersson & Hughes, 2012; Gullberg *et al.*, 2011).

Gullberg *et al.* (2011) used isogenic strains that differed only in their resistance determinants and the presence of a *yfp* or *cfp* gene encoding a yellow or cyan fluorescent protein respectively. These strains could then be sorted using fluorescence activated cell sorting (FACS) to determine how the proportion of resistant and susceptible strains had changed (Gullberg *et al.*, 2011). They found that tetracycline concentrations of 15 ng/mL

were sufficient to cause selection in favour of a resistant *S. enterica* (Gullberg *et al.*, 2011. This is similar to the concentration used in the competition experiments with *E. coli* (50 ng/mL). It is possible that if the competition experiment described in this thesis were carried for a longer period of time, with a more sensitive detection method, selection might be seen at lower antibiotic and herbicide concentrations. This would further increase the number of potential environments where bacteria could come into contact with low levels of antibiotics and herbicides that can cause selection in favour of resistant bacteria.

It has also been shown that chemicals other than antibiotics can help to maintain plasmids harbouring genes conferring resistance to both antibiotics and other biocides such as heavy metals (Gullberg *et al.*, 2014; Heinemann *et al.*, 2000). Very low antibiotic and heavy metal levels, such as those found in polluted environments, were sufficient to maintain the plasmids (Gullberg *et al.*, 2014). At high enough concentrations herbicides reduce the growth of bacteria. These concentrations vary between species; for example Roundup was more toxic to *S. aureus* than it was to *E. coli* or *S. enterica* (Section 2.4.1). It was observed in the tetracycline and Roundup competition 3 that, as the herbicide concentration increased, a slight selection in favour of the resistant bacteria was detectable. It is possible that the mechanisms that provide resistance to antibiotics, for instance efflux pumps, may also increase the tolerance of bacteria to herbicides. If this were the case, it is possible that antibiotic resistance genes could be sustained in environments containing high herbicide concentrations as they make resistant strains more fit. However, in order to properly test this hypothesis, more competition experiments would need to be carried out using higher herbicide concentrations to see if there is any significant difference in selection in favour of the resistant strain.

Competition experiments where the resistant strain is present at a lower starting proportion than the susceptible strain would also be interesting to perform. This would create conditions more similar to what is observed in the environment where mutants are normally present at low frequencies. If the resistant strain is still able to increase in proportion to a similar degree, this would provide further evidence that herbicides and antibiotics can have strong effects on the strength of selection in favour of acquired resistance.

The experiments in this chapter focused on two combinations of herbicide and antibiotic that caused a decrease in the antibiotic tolerance of *E. coli*. This decrease in tolerance meant that resistant strains were more fit and increased in proportion within a heterogeneous population. The decreased tolerance phenotype was not restricted to these combinations or this species. It is likely that selection in favour of resistant bacteria could also occur in other herbicide and antibiotic combinations and in different species. Chemicals other than herbicides that also caused decreased antibiotic tolerance would also be likely to exert a similar selective pressure.

Chapter 5

Summary and Future Directions

The work in this thesis expanded upon previous work that herbicides could induce temporary increases and decreases in the antibiotic tolerance of *E. coli* and *S. enterica* (Kurenbach et al., 2015). In particular, the species range was expanded to include the Gram-positive *S. aureus.* The change in antibiotic tolerance was measured following exposure to 24 antibiotic and herbicide combinations. The three herbicides and five antibiotics used in the previous study were tested along with three new antibiotics that are used to treat *S. aureus* infections, oxacillin, fusidic acid and vancomycin.

The next phase of this study was to test hypotheses on how these adaptive changes in antibiotic tolerance could lead to shifts in the population frequency of increased resistance to antibiotics. Adaptive resistance was shown to cause an increased frequency of acquired resistance in two herbicide and antibiotic combinations, ciprofloxacin + Kamba and ciprofloxacin + Roundup. Herbicide-induced decreases in antibiotic tolerance were shown to cause selection in favour of resistant bacteria in a heterogeneous population in two herbicide and antibiotic combinations, tetracycline + Roundup and streptomycin + Kamba

Antibiotic resistance is becoming an increasingly serious problem worldwide with experts predicting a 'post-antibiotic era' within the next 30 years if nothing is done to combat the spread of resistance (The Review on Antimicrobial Resistance, 2016; World Health Organization, 2014). Antibiotic resistant bacteria are responsible for hundreds of thousands of deaths worldwide every year and are commonly associated with increased morbidity in

patients who survive (The Review on Antimicrobial Resistance, 2016; World Health Organization, 2014).

Antibiotics are used for many purposes including the treatment of infections, crop dusting and to promote growth in farmed animals (Ghosh & LaPara, 2007; Kemper, 2008; McEwen & Fedorka-Cray, 2002). The continued and increasing use of antibiotics has contributed to the development and spread of resistant bacteria. However, bacteria come into contact with many chemical types that are not antibiotics but can still influence their antibiotic tolerance (Heinemann *et al.*, 2000). Chemicals such as salicylate (Rosner, 1985), biocides used in the food industry (Molina-Gonzalez *et al.*, 2014) and non-antibiotic pharmaceuticals (Kristiansen *et al.*, 2010) have all caused changes to the antibiotic tolerance of bacteria. Commercial herbicide formulations and their active ingredients are also a part of that list (Kurenbach *et al.*, manuscript in preparation; Kurenbach *et al.*, 2015).

I determined the concentrations of three herbicide formulations that were toxic to *S. aureus,* with Roundup showing the highest level of toxicity. However, glyphosate, the active ingredient of Roundup, does not inhibit the EPSPS enzyme of *S. aureus* (Priestman et al., 2005) raising questions about what component of Roundup is so toxic to *S. aureus.* Sub-lethal concentrations of these herbicides caused increases and decreases in the antibiotic tolerance of *S. aureus*. 2,4-D had no significant effect on the response of *S. aureus* to vancomycin or ciprofloxacin but caused an increase in tolerance to chloramphenicol, tetracycline, oxacillin and fusidic acid. 2,4-D also caused a decreased tolerance to ampicillin and kanamycin. Kamba caused an increase in to tolerance to fusidic acid, kanamycin, oxacillin, and tetracycline. Kamba had no effect on the tolerance of *S. aureus* to ampicillin, chloramphenicol and vancomycin but did cause a decrease in tolerance to ciprofloxacin.

Roundup had no effect on the response of *S. aureus* to ciprofloxacin and chloramphenicol but caused an increase in tolerance to kanamycin and vancomycin. Roundup also caused a decrease in the tolerance of *S. aureus* to ampicillin, fusidic acid, oxacillin and tetracycline.

The minimum herbicide concentrations that could induce these responses were determined for all significant combinations. *The herbicide concentrations used were all below application rates and in some cases were within the maximum residue limits allowed on human food and animal feed.* The lowest concentration of herbicide required to induce an antibiotic response in *S. aureus* was 10 ppm ae for ampicillin and tetracycline. The minimum Kamba and 2,4-D concentrations necessary to induce an antibiotic response was 300 and 50 ppm ae respectively.

Further work in this area would be to determine whether *S. aureus* responds in the same manner to the purified herbicide active ingredients as it does to the commercial formulations. Other formulation components such as surfactants could also be tested. Fusidic acid had the largest change in MIC following herbicide addition and is often prescribed as a cream, which can contain surfactants. There are other antibiotics that could be tested for instance, mupirocin, which is a topical antibiotic commonly used to treat *S. aureus* infections in New Zealand (Heffernan *et al.*, 2015). This herbicide-induced antibiotic response is likely to occur in a broad range of species and there are probably many other biocides that are capable of inducing a similar response. There is no clear pattern thus far that accounts for all responses observed so each combination of interest would need to be tested individually.

Adaptive ciprofloxacin resistance in *S. enterica* caused by exposure to Kamba or Roundup was shown to lead to acquired ciprofloxacin resistance. *S. enterica* was exposed to

ciprofloxacin and each herbicide for 48 hours and the frequency of resistant bacteria determined. This frequency was compared to herbicide only and LB controls. The ciprofloxacin and Kamba treatment caused a significant increase in the frequency of resistant bacteria compared to both controls with resistant bacteria present at a frequency over 100 times higher than either control. The ciprofloxacin and Roundup treatment similarly had resistant bacteria present in frequencies 100 times higher than the controls and was significantly different to the LB treatment. Several isolates were saved from each treatment and in future it would be interesting to determine how resistant each isolate is to ciprofloxacin. The mechanisms of this resistance could also be determined using whole genome sequencing. Another area where future work could be performed is determining the number of generations that the adaptive response will persist for once the herbicide is removed from the environment.

The final area this thesis covered was how decreases in antibiotic tolerance could lead to selection in favour of resistant bacteria in a heterogeneous population. Isogenic strains of bacteria were mixed in equal proportions in a range of conditions and the change in proportion over time was used to calculate the strength of selection in favour of the resistant strain. Conditions including herbicides and antibiotics had significantly increased selection in favour of resistance while the herbicide only and antibiotic only conditions showed no significant changes in selection strength. Kamba concentrations of 183 ppm ae and 1371 ppm ae in combination with streptomycin caused strong selection in favour of the resistant strain. 311 ppm ae of Roundup caused a similar effect on selection in favour of a tetracycline resistant strain.

There are alternative methods of measuring the change in proportion of a culture of two different strains that are more sensitive than those used in this thesis. For instance, strains could be labelled with genes encoding a different fluorescent protein to allow separation and counting cells by FACS (Gullberg *et al.*, 2011). This method may be able to detect smaller shifts in the proportion of a genotype and could prove that even lower herbicide and antibiotic concentrations can cause selection in favour of resistance. Another potential experiment would be to perform the competitions with the resistant strain starting at a lower proportion than the susceptible strain, as this would be a more likely scenario to occur in the environment.

There are many environments in which bacteria can come into contact with low levels of both herbicides and antibiotics including agricultural and urban soil, waterways, and on or within humans and animals. I have shown that herbicides can induce a range of new adaptive responses of *S. aureus* and that previously observed adaptive responses of *E. coli* and *S. enterica* can lead to an increased prevalence of acquired resistance

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